IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Miri Seiberg, et al.	
Serial No. 09/206,249	METHODS FOR REGULATING PHAGOCYTOSIS AND ICAM-1 EXPRESSION
Filed: December 7, 1998	
Art Unit: 1654	
Examiner: M. Meller	
Attorney Docket No.: JBP 438	

DECLARATION OF MIRI SEIBERG, PH.D.

I, Miri Seiberg, am a Distinguished Research Fellow in the Skin Research Center at Johnson & Johnson Consumer Companies, Inc. My education includes a Ph.D. in Molecular Biology from The Weizmann Institute of Science, Rehovot, Israel, in collaboration with Princeton University, Princeton, NJ and a B. S. in Life Sciences from Tel-Aviv University, Tel-Aviv, Israel. My curriculum vitae is attached hereto as Exhibit 1.

1. All protein molecules are composed of polypeptide chains of α-amino acids. Proteins are defined by both (1) their chemical structure, which includes its substituent amino acids as well as their unique conformation and (2) their biological function. A protein's biological function or activity requires the presence of both its chemical structure and conformation. (Biochemistry, A. L. Lehninger, 1975, p. 62- 66).

2. Proteins are said to be "denatured" when their physical and physiological properties are changed such that they lose their activity. Such change is generally due to a change in a protein's chemical structure and/or conformation. Protein denaturation and the consequent loss of biological activity are not related to the source of the protein or to their origin, and are described in biochemistry textbooks (e.g. Biochemistry, A. L. Lehninger, 1975, p.62-63).

3. Those knowledgeable about protein activity at the time the invention was made were aware that proteins are denatured in the presence of organic solvents. The effect of organic chemicals on protein denaturation has been studied for decades. A 1975 publication from Matveev describes the dependence of denaturation time on organic solvent concentrations. Extraction with organic solvents was shown to denature many proteins (Sikorski and Naczk, 1981). In 1984, Benedek et al measured the kinetics of denaturation of several proteins, including soybean trypsin inhibitor (STI), as a function of the organic modifier employed. Khmelnitsky et al (1991) documented the denaturation of several proteins by a broad series of organic solvents of different nature. van Erp et al (1991) developed a theoretical model, based

on a generally accepted notion that the destruction of the protein hydration shell is one of the main reasons for protein denaturation by organic solvents. These studies document that proteins (including STI) are denatured in the presence of organic solvents. Copies of the foregoing references are attached hereto as Exhibit 2.

4. Genistein is an isoflavone. Those knowledgeable of the process of isolation and purification of isoflavones are aware that the extraction of genistein from soybeans requires organic solvents. This solvent extraction knowledge was used, e.g. in United States Patent 5,679,806, in purifying isoflavones by using three steps of solvent extraction.

5. During such organic extraction processes, STI (and all other proteins) separates into the aqueous phase, which is removed from the final isoflavone preparation. As indicated above, residual STI that might have contaminated the organic phase of the genistein preparation would be denatured, and therefore inactive due to the presence of organic solvents. Therefore, a genistein preparation extracted from soy cannot contain even residual amounts of non-denatured and active STI.

6. A diffusion coefficient is a constant of proportionality that represents the amount of a substance diffusing across a unit area, through a unit concentration gradient, in unit time. Those knowledgeable about protein biophysical properties are aware that even decades ago it was possible to calculate and to measure the diffusion coefficients of proteins. It is known that the diffusion coefficients for biological molecules normally range from 10^{-11} to 10^{-10} m^2/s. (Fick's law of diffusion).

7. In 1946, Kunitz (attached hereto as Exhibit 3) measured the biophysical properties of STI. He found that the diffusion coefficient of STI is extremely low, therefore it was measured using a modified time unit, replacing the standard "per second" unit with a "per day" unit. Kunitz found that the diffusion coefficient of STI is 0.07-0.08 cm^2/day, at 24 degrees centigrade. It is obvious to those of ordinary skill in the art from this diffusion coefficient that STI will not diffuse out of the soybean into any soaking liquid.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dr. Miri Seiberg

7/30/08

Date

Exhibit 1

Miri Seiberg
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Education

1977 B.Sc. Biological Sciences, Tel-Aviv University, Israel.
1982 M.Sc. Biochemistry, The Weizmann Institute of Science, Israel.
1989 Ph.D. Molecular Biology, The Weizmann Institute of Science, Israel, in collaboration with Princeton University, Princeton NJ.

Employment

1982 **The Weizmann Institute of Science, Israel.**
Research assistant, Dept. of Chemical Immunology.

1982-90 **Princeton University, Princeton NJ**
1982-84 Visitor, Dept. of Biochemical Sciences.
1987-89 Visitor, Dept. of Molecular Biology.
1989-90 Post Doctoral Fellow, Dept. of Biology.

1990- 92 **Bristol-Myers Squibb PRI, Princeton NJ.**
Post Doctoral Fellow, Dept. of Macromolecular Structure.

1992- **Johnson & Johnson Family of Companies**
1992-95 Senior Scientist, Skin Biology Research Center of Pharmaceutical Research Institute, Raritan NJ.
1995-96 Staff Scientist, Dermatology R&D, Johnson & Johnson Consumer Companies, CPWW division, Skillman NJ.
1997-99 Principal Scientist, Skin Research Center, CPWW, Skillman NJ
1999-0 Research fellow, Skin Research Center, CPWW, Skillman NJ
2001 -05 Sr. Research fellow, Skin Biology TRC and LAS, CPWW, Skillman NJ
5/2005- Principal Research fellow, Skin Biology TRC and LAS, CPWW, Skillman NJ

Industrial Experience

1990- 92, Bristol-Myers Squibb PRI, Princeton NJ.

Post Doctoral Fellow, Dept. of Macromolecular Structure.

Using a rat model system for salt-induced hypertension, identified a novel gene involved in salt-induced hypertension, and demonstrated selective expression patterns.

1992-today, Johnson & Johnson Pharmaceutical Research Division

1992-95, Senior Scientist

This position involves conducting individual projects, supervising one BS/MS technician. Identified pathways involved in epidermal differentiation, hair growth and keratinocyte apoptosis. Developed relevant bioassays and screens.

Johnson & Johnson Consumer Companies, Inc.

1995-96, Staff Scientist

Directed two research scientists. Developed enzymatic, molecular and cellular assays and screens for potential drug and cosmetic activity. Involved in retinoid studies, proteases and protease inhibitors, in epidermal differentiation and hair growth.

1997-99, Principal Scientist

Head of pigmentation group. Directed research scientists and postdoctoral fellows. Horizontally directed the pigmentation technology development team. Initiated and directed molecular, cellular, and biochemical studies of pigmentation, resulting in the identification of a novel pathway that regulates skin color. Identified agents, both drugs and cosmetics, to modulate this pathway, resulting in darkening or lightening of human skin. Designed and evaluating product prototypes for biological activity and efficacy. In charge of numerous academic collaborations.

1999-00, Research Fellow

Continue heading the pigmentation team and supporting technology and product design groups in creating a line of depigmenting agents. First products available in stores. Additional responsibility in heading the hair growth efforts, introducing a new concept for delaying hair growth. Identified novel cosmetic agents with modulatory effect, demonstrated preclinical POP and initiated product development efforts. Expand responsibility for academic collaborations.

2001 - 2005, Sr. Research Fellow

Director of the Skin Biology research group, including pigmentation, hair, acne, skin aging and skin cancer teams and supporting facilities. Continue basic research and product development support in all areas. Identified a novel cosmetic for skin aging, currently under early development stages. Directed efforts in the development of a new drug for acne, based on a proprietary target, now under clinical evaluation. Continue R&D support for skin lightening technology, now sold by numerous Brands and J&J companies worldwide. Continue R&D support for delaying hair growth technology, now sold by numerous J&J companies and Brands worldwide. Received the Johnson Medal, the highest level of scientific recognition by JJ. Head of Laboratory Animal Services, incl. vivarium support for numerous J&J companies. Council member of the J&J Corporate office of Science and Technology. In charge of academic interactions and collaborations for Skin Biology and related areas. In charge of the J&J SRC training grant. Member of the mentoring team.

5/2005 to present, Principal Research Fellow

Patent applications

More than 25 patent applications in the areas of skin and hair

J&J Awards

1. Skin care council – best scientific content poster award. June 1993.
2. American Express achievement award of PRI. January 1995.
3. COSAT-CORD internship award. April 1997.
4. Skin care council – best overall poster award. June 1999.
5. COSAT excellence in science award. November 1999.
6. CPWW achievement award. January 2000.
7. Skin care council – best overall poster award. June 2001.
8. CPPW Grandview award. March 2003.
9. The Johnson Medal. Oct 2003.
10. The Mountainview award. March 2005.

Societies

1. Pan American Society of Pigment Cell Research (council member, 2001-03, member of finance committee, 2000-02, nominated for 2005 presidency elections).
2. Society of Investigative Dermatology
3. American Society of Cellular Biology
4. American Association for the Advancement of science
5. New York Academy of Science (elected 2003)

Exhibit 2

Tsitologiia. 1975 Nov;17(11):1278-82.

Denaturation time of actomyosin exposed to different chemicals

Matveev VV

The frog skeleton muscle actomyosin denaturation time dependence on the concentration of salts (NaCl and CaCl2) and organic chemicals (carbohydrates, narcotics and alcohols) was investigated. The following effects were detected: phase change in denaturation time associated with the rise of concentration of chemicals under study; actomyosin stabilization effect; coincidence of concentrations giving rise to protein stability with those increasing the survival time of isolated frog sceleton muscles in vitro (literature data); denaturation effect of alcohols used, both in high and very low concentrations.

Crit Rev Food Sci Nutr. 1981;14(3):201-30.

Modification of technological properties of fish protein concentrates.

- Sikorski ZE
- Naczk M

Fish protein concentrates are mixtures of cross-linked and aggregated molecules of different muscle proteins. The final conformation of the components of the mixtures is formed as a result of procedures applied to convert the raw materials into a product of desirable and stable sensory properties, containing less than 0.1% of lipids. To achieve this end usually extraction with hot organic solvents, mainly isopropyl alcohol and 1,2-dichloroethene, followed by air drying are employed. These conditions bring about denaturation of many of the proteins followed by aggregation of the molecules due to the interaction of reactive functional groups in extended polypeptide chains. In the final product a large proportion of hydrophobic groups is exposed to the solvent and the proteins exhibit an extremely low water affinity. Such concentrates, although valuable as protein supplements, have only limited suitability as active components of various processed foods, as they have poor technological value. They are insoluble or have a very low water dispersibility and swelling ability, do not form gels after heating, or have any significant fat-emulsifying capacity. Changing the dissociation or number of ionic groups of the molecules prior to extraction, e.g., by acidifying or acylating, can partially reduce the denaturing effect of heat and organic solvents and thus improve the functional properties of the product. An upgrading of the quality of concentrates produced by hot extraction can be achieved by partial enzymatic or chemical deaggregation, hydrolysis followed by the plastein reaction, or formation of suitable derivatives. The best results have been obtained by partial hydrolysis of acylated proteins or precipitation of the aggregated products using sodium hexametaphosphate. The functional properties of such products are comparable to those of vegetable protein isolates used as meat extenders. Various proteins of high technological value can be also obtained by enzymatic hydrolysis of the raw material, followed by separation of the lipids without organic solvent extraction. Such products, however, have a distinct odor and flavor and must be stabilized because of residual lipids.

J Chromatogr. 1984 Dec 28;317:227-43.

Kinetics of unfolding of proteins on hydrophobic surfaces in reversed-phase liquid chromatography.

- Benedek K
- Dong S
- Karger BL

As a continuation of previous studies, we present in this paper measurements on the kinetics of denaturation of papain, soybean trypsin inhibitor and lysozyme on n-butyl-bonded silica gel surfaces used in reversed-phase liquid chromatography (RPLC). In all cases, native and denatured peaks widely separated from one another are observed. The rate constants for denaturation or unfolding are determined by the measurement of the peak area of the native protein as a function of the incubation time that the species spends on the bonded-phase surface. The results reveal that a slow denaturation step occurs with a half-life of ca. 15 min. In addition, studies of denaturation as a function of the amount of 1-propanol in the initial mobile phase suggest an additional unfolding step when the protein comes in contact with the bonded-phase surface. The extent of this latter step decreases as the concentration of 1-propanol increases, further suggesting that 1-propanol sorption on the bonded stationary phase may play a role in this behavior. Other studies are conducted with alpha-chymotrypsinogen, in which injection is made after the start of the gradient. The extent of denaturation is observed to be a function of the organic modifier employed. The results of this paper provide insight into the denaturation process in RPLC and suggest approaches to minimize this behavior.

Eur J Biochem. 1991 May 23;198(1):31-41.

Denaturation capacity: a new quantitative criterion for selection of organic solvents as reaction media in biocatalysis.

- Khmelnitsky YL
- Mozhaev VV
- Belova AB
- Sergeeva MV
- Martinek K

A. N. Bakh Institute of Biochemistry, Moscow, USSR.

The process of reversible denaturation of several proteins (alpha-chymotrypsin, trypsin, laccase, chymotrypsinogen, cytochrome c and myoglobin) by a broad series of organic solvents of different nature was investigated using both our own and literature data, based on the results of kinetic and spectroscopic measurements. In all systems studied, the denaturation proceeded in a threshold manner, i.e. an abrupt change in catalytic and/or spectroscopic properties of dissolved proteins was observed after a certain threshold concentration of the organic solvent had been reached. To account for the observed features of the denaturation process, a thermodynamic model of the reversible protein denaturation by organic solvents was developed, based on the widely accepted notion that

an undisturbed water shell around the protein globule is a prerequisite for the retention of the native state of the protein. The quantitative treatment led to the equation relating the threshold concentration of the organic solvent with its physicochemical characteristics, such as hydrophobicity, solvating ability and molecular geometry. This equation described well the experimental data for all proteins tested. Based on the thermodynamic model of protein denaturation, a novel quantitative parameter characterizing the denaturing strength of organic solvents, called the denaturation capacity (DC), was suggested. Different organic solvents, arranged according to their DC values, form the DC scale of organic solvents which permits theoretical prediction of the threshold concentration of any organic solvent for a given protein. The validity of the DC scale for this kind of prediction was verified for all proteins tested and a large number of organic solvents.

European Journal of Biochemistry, Vol 202, 379-384, Copyright © 1991 by Federation of European Biochemical Societies

The effect of water content and nature of organic solvent on enzyme activity in low-water media. A quantitative description

SH van Erp, EO Kamenskaya and YL Khmelnitsky
A. N. Bakh Institute of Biochemistry, Moscow, USSR.

A simple theoretical model was suggested to describe quantitatively the effect of water content and nature of organic solvents on catalytic behavior of enzymes suspended in low-water media. The model was based on a generally accepted notion that the destruction of the protein hydration shell is one of the main reasons for protein denaturation by organic solvents. The validity of the model was confirmed by the example of catalytic behavior of immobilized laccase suspended in water/organic mixtures of different compositions. In addition, the results were used to demonstrate that the effect of organic solvents and/or water content on catalytic behavior of enzymes in low-water media can be adequately assessed only in terms of the full kinetic description based on properly determined Vm and Km values.

Process for the isolation and purification of isoflavones

United States Patent 5679806 (Filed 02/24/1995, granted 10/21/1997)

Abstract: The present invention relates to a process for the isolation and purification of isoflavones from a number of different biomass sources. More particularly, the present invention relates to a three-step process whereby a biomass containing isoflavones with a solvent thereby forming an extract that is subsequently fractionated using a reverse phase matrix in combination with a step gradient elution, wherein the resulting fractions eluted from the column contain specific isoflavones that are later crystallized. The purified isoflavone glycosides may then be hydrolyzed to their respective aglycone.

Published March 20, 1947

CRYSTALLINE SOYBEAN TRYPSIN INHIBITOR

II. General Properties

By M. KUNITZ

(*From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

(Received for publication, October 21, 1946)

The isolation of a crystalline trypsin inhibitor from soybean has been reported in previous publications (1). This paper deals with some of the properties of the new crystalline protein and with the mechanism of its inhibiting action on trypsin and chymotrypsin.

The soybean inhibitor is a protein of the globulin type. It is precipitated by trichloracetic acid and is non-diffusable through collodion or cellophane membranes. Its light absorption spectrum is that of a typical protein with a maximum at 280 mμ and a minimum at 252 mμ. The protein contains less than 0.01 per cent phosphorus and is free of carbohydrate. It acts as an inhibitor only when it is in its native state; denaturation of the soy protein by heat, acid, or alkali is accompanied by a loss in its inhibiting power.

The action of the native soybean protein as a trypsin inhibitor is due to its combination with trypsin to form an irreversible stoichiometric compound. The combination is apparently instantaneous.

The soy protein inhibits slightly the proteolytic action of chymotrypsin, but unlike that of trypsin the inhibition is due to the formation of a loose reversible compound of the type described by Northrop (2) for the combination between pepsin or trypsin with crude inhibitors. The reaction between chymotrypsin and the soybean inhibitor was found to agree with the law of mass action, for a reversible uni-unimolecular reaction.

Crystalline soybean protein, if denatured, is readily digestible by pepsin, by chymotrypsin, or by trypsin.

Crystalline soybean inhibitor has no inhibiting effect either on the proteolytic activity or on the milk-clotting ability of pepsin.

EXPERIMENTAL

Test of Purity of Crystalline Soybean Trypsin Inhibitor[1]

1. Effect of Recrystallization.—The principal steps in the procedure for the

[1] For the sake of brevity the terms "soy inhibitor" and "soy protein" are frequently used in the text instead of the full expression "crystalline soybean trypsin inhibitor."

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Published March 20, 1947

isolation of the crystalline soybean inhibitor are the following: (1) Extraction of soybean meal in 0.25 N H_2SO_4. (2) Adsorption of the inhibitor from the acid extract on bentonite and elution with 5 per cent solution of pyridine in water. The pyridine is removed by dialysis. (3) Precipitation of the inhibitor in amorphous form at pH 4.65. This step is repeated twice. (4) Crystallization at pH 5.0 and 35°C. The extent of purification during the various stages of preparation is shown in Table I.

The material reaches its highest purity after two crystallizations, as shown by measurements of inhibiting activity and also by the Molisch test for carbohydrate impurities. The specific activity of the second mother liquor no longer differs from that of the second crystals. Further crystallization does

TABLE I

Effect of Crystallization on the Purity of Soybean Inhibitor

1000 gm. Nutrisoy XXX flakes.

Preparation	Volume	Total yield	Specific activity	Molisch test for carbohydrate
	ml.	*units of trypsin inhibitor*	*units per mg. protein*	
Acid extract	5,000	10,000	0.38	++++
Dialyzed bentonite elute	475	8,000	0.85	
2nd amorphous precipitate pH 4.65	8–10 gm.	3,000	1.00	
1st crystals	4 gm.	1,500	1.03	?
1st mother liquor			0.88	+
2nd crystals			1.05	−
2nd mother liquor			1.02	−

not have any significant influence on the specific activity of the crystalline protein.

2. Solubility Test for Purity.—The purity of a sample of several times crystallized soybean inhibitor was tested by measuring the solubility of the material in 0.1 M acetate buffer pH 4.6 at 5°C. in the presence of increasing amounts of solid protein in suspension.

The usual procedure of stirring increasing amounts of crystals in a definite volume of solvent until equilibrium is reached was found unsuitable for the soy inhibitor, since stirring brought about gradual denaturation of the protein. It was found more satisfactory to use solutions of various concentrations of the protein and then to bring about saturation by adjusting the pH to that of the isoelectric point of the material. Rapid equilibrium is thus established between the solid phase in form of amorphous precipitate and the saturated solution. The details of the procedure are as follows:

Experimental Procedure.—5 gm. of three times crystallized soy inhibitor were dissolved in 50 ml. 0.1 M sodium acetate at 5°C. The protein was then precipitated in

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amorphous state by adding rapidly 50 ml. 0.1 M acetic acid; the precipitate was filtered with suction on hardened paper at about 5°C. The filter cake was redissolved in 50 ml. 0.1 M sodium acetate and reprecipitated again with 50 ml. 0.1 M acetic acid. The precipitation was repeated once more. The protein concentration of the second and third filtrates was determined and found to be practically identical and equal in each case to about 1.5 mg. per ml. The final precipitate was resuspended at 5°C. in 100 ml. of a mixture of equal parts of 0.1 M sodium acetate and 0.1 M acetic acid. Various amounts of suspension, from 0.1 to 5 ml., were then distributed in test tubes containing 5 ml. of cold 0.1 M sodium acetate. This brought about complete solution of the precipitate. The clear solutions were then poured back and forth into test tubes containing 5 ml. cold 0.1 M acetic acid. A precipitate of amorphous protein was immediately formed in every tube except in those containing very small amounts of the original suspension. Samples of 1 ml. were taken for total protein determination and the remaining material was centrifuged at 5°C. The protein concentration of the supernatant solutions was then determined.

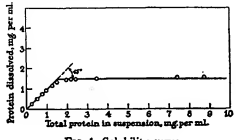

FIG. 1. Solubility curve.

The results are shown in Fig. 1. The solid lines represent the theoretical phase rule curve for a pure substance. According to the phase rule the solubility of a pure substance is independent of the amount of solid phase of the substance present in suspension. The first four points fall on the 45° line since there was complete solution in the mixtures corresponding to those points. The other points lie close to the theoretical horizontal line except for those near the intersection of the straight lines. This irregularity has been observed frequently in the solubility curves of a number of other crystalline proteins and it may be due to the presence of small amounts of denatured protein formed during the equilibration process. The solubility experiment as a whole shows that the material is free of any impurities which can be removed by washing. It does not preclude however the possibility of the presence of an impurity which has a tendency to form a solid solution with the bulk of the material.

Reaction between Crystalline Soybean Inhibitor and Crystalline Trypsin

Addition of increasing amounts of soy inhibitor to a solution of trypsin decreases the proteolytic activity of the trypsin in direct proportion to the amount of soy inhibitor added. Pure soy inhibitor counteracts approximately an equal

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weight of pure trypsin. The inhibition is apparently instantaneous and is independent, within a wide range, of the pH of the solution.

The quantitative relationship between the amount of soy inhibitor added and the amount of trypsin inhibited is shown in Figs. 2 and 3.

The amount of trypsin inhibited is directly proportional to the amount of inhibitor used and is independent of the total concentration of trypsin in the inactivation mixture.

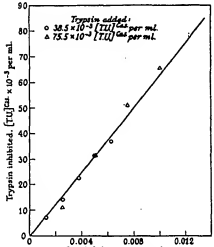

FIG. 2

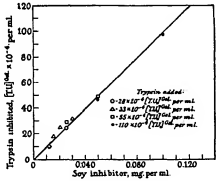

FIG. 3

FIG. 2. Effect of soy inhibitor on the digestion of casein by trypsin.
FIG. 3. Effect of soy inhibitor on the digestion of gelatin by trypsin.

Experimental Procedure.—1 ml. samples of a solution of 50 γ crystalline trypsin per ml. 0.0025 M HCl were mixed with 1 ml. samples containing increasing amounts of soy inhibitor dissolved in 0.0025 M HCl. The amount of inhibitor varied from 0 to 50 γ per ml. in steps of 10 γ. 1 ml. of each mixture was then added to 1 ml. samples of 1 per cent casein pH 7.6 and the tryptic activity was determined as described in the section on Methods. The same experiment was repeated with samples of a stock solution containing 25 γ of trypsin per ml. The results of the two experiments are given in Fig. 2.

The direct proportionality between the amount of inhibitor used and the amount of trypsin inhibited, independently of the total concentration of trypsin in solution, is also shown in Fig. 3. In this case the inactivation mixture was at pH 7.6 and the inhibition was measured by the gelatin formol titration method (see Methods).

The amount of trypsin inhibited per unit weight of inhibitor, when expressed in tryptic units, is independent of the purity of the preparation of trypsin used

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and it corresponds approximately to a weight of pure trypsin equal to the weight of inhibitor used. It appears that the reaction between soy inhibitor and trypsin is of the ionic type similar to neutralization of H ion by OH ion. The reaction cannot be reversed either by dilution or by change of pH.

Isolation of a Crystalline Compound of Trypsin and Soybean Inhibitor

A crystalline protein has been isolated from a solution containing crystalline trypsin and crystalline soy inhibitor. The new protein is composed of about equal weights of trypsin and inhibitor proteins. It is inert when added to a solution of casein or gelatin, but it does show either tryptic or inhibitory activity when denatured selectively. The method of isolation of the compound and a description of some of its properties are given in the subsequent paper.

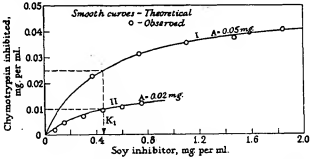

FIG. 4. Effect of soy inhibitor on clotting of milk by chymotrypsin.

Reaction between Chymotrypsin and Soybean Inhibitor

Soy inhibitor exerts a slight inhibiting effect on the proteolytic and the milk-clotting activities of chymotrypsin. The relationship between the amount of inhibitor used and the amount of chymotrypsin inhibited as tested on the ability of chymotrypsin to clot milk is shown in Fig. 4. The plotted curves differ strikingly from those obtained for trypsin (Figs. 2 and 3). The amount of chymotrypsin inhibited per unit weight of inhibitor is small compared to that of trypsin and it decreases rapidly with the relative proportion of total inhibitor and chymotrypsin mixed. The data on the amount of chymotrypsin inhibited when 20 γ per ml. were used fall on a lower curve than the data for 50 γ chymotrypsin per ml. The lack of proportionality between the amount of chymotrypsin inhibited and the soy inhibitor used holds true also for the effect on the digestion of casein, as shown in Fig. 5.

The type of curves obtained is similar to that of the curves obtained by Northrop (2) in his studies of the effect of crude inhibitors on pepsin and trypsin and suggests the same mechanism, namely, that the reaction between the soy inhibitor and chymotrypsin is of the reversible type obeying the law of mass

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action so that there is always an equilibrium between the concentration of the product of the reaction and the concentrations of the reactants in solution.

An analysis of the data is simplified by the fact that the total amount of the inhibitor in all the solutions used is large compared with the amount of inhibitor combined with chymotrypsin so that the concentration of free inhibitor equals approximately that of the total inhibitor taken. It is assumed here, as in the case of Northrop's experiments, that the reaction is uni-unimolecular so that one molecule of chymotrypsin combines reversibly with one molecule of inhibitor to form one molecule of an addition compound.

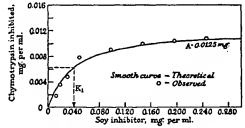

FIG. 5. Effect of soy inhibitor on the digestion of casein by chymotrypsin.

Let M_a and M_b be the molecular weights of chymotrypsin and the soy inhibitor proteins respectively. Let also A and B equal total weights and a and b equal weights of the free chymotrypsin and inhibitor in solution in volume V, then at equilibrium we have, in accordance with the law of mass action for a reversible reaction,

$$\frac{a}{M_a V} \times \frac{b}{M_b V} = K \frac{A - a}{M_a V} \tag{1}$$

$\frac{A - a}{M_a V}$ being the concentration of the compound formed which is numerically the same as the concentration of the inhibited chymotrypsin.

K = equilibrium constant.

Since $b = B$ (approximately) Equation 1 becomes

$$\frac{a}{A - a} = \frac{V}{B} K_1 \tag{2}$$

where $K_1 = KM_b$ and is equal numerically to the value of $\frac{B}{V}$ at 50 per cent inhibition, *i.e.*, when $\frac{a}{A - a} = 1$. Equation 2 can be also written as

$$\frac{A - C}{C} = \frac{VK_1}{B}$$

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Solving for C we get

$$C = \frac{AB}{VK_1 + B} \tag{3}$$

Equation 3 was used to calculate the values of C for the theoretical curves given in Figs. 4 and 5 for the relationship between C = the weight of chymotrypsin combined and B = the total weight of inhibitor used, V being equal to 1, since the weights given

TABLE II

Calculation of the Theoretical Curves for the Inhibition of Chymotrypsin by Soybean Inhibitor

Curve	A	K_1	B	C Calculated	C Observed	Equation
	γ		γ	γ	γ	
Fig. 4, I	50	450	367	22.5	22.7	
			734	31.0	31.2	
			1100	35.5	35.5	$C = \frac{50B}{450 + B}$
			1470	38.2	37.4	
			1840	40.2	40.6	
Fig. 4, II	20	450	75	2.9	2.0	
			150	5.0	4.6	
			300	7.0	7.2	$C = \frac{20B}{450 + B}$
			450	10.0	9.1	
			600	11.4	10.5	
			750	12.5	12.1	
Fig. 5	12.5	40	12.2	2.9	1.7	
			18.3	3.9	3.5	
			30.5	5.4	4.8	
			49.0	6.9	7.8	
			98	8.9	9.1	$C = \frac{12.5B}{40 + B}$
			147	9.9	10.0	
			196	10.4	10.5	
			245	10.7	10.8	

were expressed per unit volume. The value of K_1 was read in each case at $C = 0.5\,A$ on a preliminary smooth curve drawn between the experimental points in the same region.

The calculated values of C are given in Table II. They are identical, within experimental error, with the observed data given in the same table. The experimental results are in agreement with the theoretical assumption that the mechanism of inhibition of chymotrypsin by soybean inhibitor consists in the formation of a uni-unimolecular compound in equilibrium with free chymotrypsin and soy inhibitor in solution. Equation 3 shows that the amount of chymotrypsin inhibited per unit weight of soy inhibitor is proportional to the total

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amount of chymotrypsin in solution and is decreased with the increase in amount of inhibitor used and with dilution; in the case of trypsin, the amount of trypsin inhibited per unit weight of soy inhibitor is constant and is equal approximately to the weight of inhibitor used, independent of the total concentration of trypsin or inhibitor.

It is to be noticed that the value of K_1 while identical in Fig. 4 for curve I and curve II, differs from the value of that constant given in Fig. 5. The concentrations of the reactants given in all cases have been expressed in weights per milliliter of the inactivation mixtures, without considering the further dilution and further changes caused on addition of samples of 1 ml. of the mixture to the substrates used for activity measurements. The sample was added

TABLE III

Effect of Soy Inhibitor on Clotting of Milk by Pepsin

Pepsin-inhibitor mixtures					
Pepsin 4 γ per ml. 0.1 M acetate buffer pH 5.0, *ml.*....	0.5	0.5	0.5	0.5	0.5
Soy inhibitor 1 mg. per ml. acetate buffer pH 5.0, *ml.*.	0	0.2	0.3	0.4	0.5
Made up to 1 ml. with 0.1 M acetate buffer pH 5.0. Mixtures left in room for 10 minutes, then 0.5 ml. of each added to 2.5 ml. of 20 per cent Klim milk in 0.1 M acetate buffer pH 5.0 at 36°C. and time of clotting observed.					
Clotting time, *min.*....	3.5–4.9	3.5	3.5	3.3	3.0
Repeated with same pepsin inhibitor mixtures after standing at 25°C. for 3 hrs.					
Clotting time, *min.*....	3.5	3.1	2.9	2.9	2.8

to 2.5 ml. of 20 per cent solution of dry Klim milk of pH 5.8 in the experiments on milk clotting or to 1 ml. of 1 per cent solution of casein pH 7.6. Hence the difference in the equilibrium constant in the two cases.

The thoretical Equation 2 has been checked directly by substituting the experimental data and solving for K_1; approximately constant values of K_1 were obtained equal to those given in Table II.

Effect on Pepsin

Soy inhibitor has no inhibiting effect on pepsin, either on its proteolytic activity at pH 2.0 or on its ability to clot milk at pH 5.8. At pH 2.0 soy inhibitor is digestible by pepsin. The experiment on clotting of milk is given in Table III.

The Globulin Nature of Soy Protein

A globulin is defined as a protein which in its native state has a minimum solubility at the isoelectric point; the solubility increases in the presence of

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salt. In accordance with this definition, the soy inhibitor protein is a typical globulin being least soluble at its isolectric point in the absence of salt. Addition of salt however increases its solubility.

(a) Solubility and pH. Isoelectric Point.—

Experimental Procedure.—Samples of 0.1 ml. of a stock suspension of 10 mg. of crystals of soy inhibitor per ml. of H_2O of pH about 4.5 were added to 10 ml. 0.02 M acetate buffers of varied pH. The pH and turbidity of the various mixtures were measured after standing for several hours at about 25°C. The cataphoretic mobility of the crystals in the same mixtures was measured the same day. The results are given in Table IV.

TABLE IV

Isoelectric Point of Crystalline Soybean Trypsin Inhibitor and Solubility

pH (quinhydrone electrode)	4.15	4.27	4.49	4.70	4.81	4.87	5.00	5.12	5.34	5.50	5.65	5.80
Light absorption measured at 600 mμ												
Optical density	0.095	0.150	0.196	0.195	0.135	0.070	0.045	0.043	0.040	0.034	0.038	0.032
Cataphoretic mobility: 1 extra drop of concentrated suspension of crystals added to the solution above pH 4.80												
Mobility in μ per sec. per volt per cm.	+1.75	+0.88	− trace	−1.17	−1.45	−1.82	−2.20	−2.20	−2.9	−3.1		

TABLE V

Effect of Salts on Solubility of Soy Inhibitor at pH 4.5

3 ml. samples of a stock suspension of 100 mg. of soy inhibitor in 20 ml. water at pH 4.5 were mixed with 3 ml. of various salt solutions in 0.02 M acetate buffer pH 4.5.

Final salt solutions	0.01 M acetate buffer pH 4.5	0.1 M acetate buffer pH 4.5	0.1 M NaCl	0.05 M Na_2SO_4	0.05 M $MgSO_4$	0.1 M $MgSO_4$
			All in 0.01 M acetate buffer pH 4.5			
Suspensions left 18 hrs. at 10°C., then 1 hr. at 25°C. Centrifuged clear at 2500 R.P.M. for 10 min.						
Mg. protein per ml. supernatant (Determined by measuring optical density at 280 mμ)	0.73	0.95	1.57	1.51	1.565	2.01

The crystals of the soy inhibitor are least soluble at the point of minimum cataphoretic mobility, the isoelectric point, which is at pH 4.5.

(b) Effect of Salts on the Solubility of Soy Inhibitor at the Isoelectric Point of the Protein.—The solubility of the crystals of soy inhibitor at its isoelectric point is considerably increased in the presence of salts. This is shown in Table V. The crystals are more soluble in the presence of magnesium ions than in the presence of an equivalent concentration of sodium ions.

Stability and Denaturation of Soy Inhibitor

Crystalline soy inhibitor is stable in the range of pH of 1–12 when dissolved in dilute buffer solution and stored at temperatures below 40°C. At higher

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temperatures and in stronger acid or alkaline solutions the protein is gradually denatured, as evidenced by a decrease in its solubility at the isoelectric point or in salt solutions. The gradual denaturation of the protein is accompanied by a corresponding loss in its ability to inhibit the action of trypsin.

1. Denaturation in 0.1 M NaOH.—

A solution of 0.1 M NaOH containing 2.5 mg. soy inhibitor per ml. was left at 36°C. 2 ml. samples were taken after various intervals of time, neutralized with 2 ml. of 0.1 M HCl, and analyzed for denatured protein and inhibiting activity. The amount of denatured protein was determined by mixing 2 ml. of the neutralized solution with 4 ml. of 0.1 M acetate buffer pH 4.5 and centrifuging the precipitate formed after

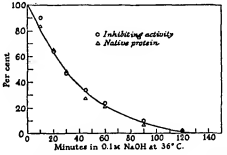

FIG. 6. Denaturation of soy inhibitor in 0.1 M NaOH at 36°C.

standing about 1 hour at room temperature. The precipitate consisted of denatured protein while the supernatant solution contained native protein. The concentration of protein in the supernatant solution was measured spectrophotometrically at 280 mμ. The inhibiting activity was determined on samples of the neutralized solution without addition of acetate buffer.

The results of the experiment are given in Fig. 6. The gradual loss in native protein is accompanied by a corresponding percentage loss in inhibiting activity.

Denaturation in 0.1 M HCl at 50°C. or higher gave results similar to those obtained on denaturation in 0.1 M NaOH.

2. Reversible Denaturation by Heat.—Soy inhibitor protein like many other proteins becomes denatured when heated in dilute acid or alkaline solution at temperatures above 40°C. The denaturation in the absence of salts is not accompanied by any visible precipitation of denatured material. The denatured protein is readily precipitable on addition of salt or on adjusting the pH of the heated solution to that of the isoelectric point. The denaturation is reversed on cooling. Prolonged heating however brings about permanent denaturation. The following experiments show that denaturation on heating and also the reversal of the denaturation on cooling as well as irreversible denaturation are accompanied by a corresponding loss or gain in the inhibiting activity.

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(*a*) *Denaturation (Reversible) at 70°C.*—Samples of 2 ml. 0.1 per cent solution of soy inhibitor of pH about 3.0 (in 0.0006 M HCl) were placed in a water bath at 70°C., removed at various times, cooled to about 5°C., and mixed with 4 ml. of 0.15 M acetate buffer pH 4.5. The precipitates formed were centrifuged after standing 1 hour in the room. The supernatant solutions were analyzed for protein and inhibiting activity.[2] The results are shown in Fig. 7.

(*b*) *Reversal of Denaturation at 30°C.*—25 ml. of a 0.25 per cent solution of crystalline soy inhibitor in 0.0025 M HCl were heated at 80°C. for 5 minutes, then transferred to a water bath at 30°C. Samples of 1 ml. were mixed at various times with 5 ml. 0.06 M acetate buffer pH 4.5 and treated as described in (*a*). The results are shown in Fig. 8.

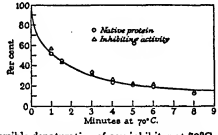

FIG. 7. Reversible denaturation of soy inhibitor at 70°C. and pH 3.0.

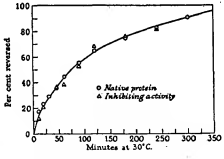

FIG. 8. Reversal of denaturation.

(*c*) *Irreversible Denaturation at 90°C.*—Samples of 1 ml. solution of 0.5 per cent crystalline soy inhibitor in 0.0025 M HCl were heated at 90°C. for various lengths of time and stored at 20°C. for 18 hours. Each sample was then mixed with 5 ml. 0.06 M acetate buffer pH 4.5 and treated as described in (*a*).

The results are shown in Fig. 9.

The denaturation by heat and also the reversal of denaturation on cooling proceed at a measurable rate. At temperatures above 40° and below 60°C. denaturation and the reversal of denaturation proceed until a point of equi-

[2] Activity measurements when done on the uncentrifuged suspension gave higher values, possibly because of reversal of denaturation in the digestion mixture at pH 7.6.

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librium is reached between the amount of native and denatured protein in solution. The equilibrium values depend on the temperature and the pH of the solution. Studies of the kinetics and the thermodynamics of reversible denaturation of crystalline soybean inhibitor protein will be described in a separate paper.

Digestion of Soy Inhibitor Protein by Proteolytic Enzymes

1. Digestion by Pepsin.—Crystalline soy inhibitor protein, if denatured, is readily digestible by pepsin in slightly acid solution. Native soy inhibitor protein is hardly affected by pepsin at pH 3.0. However, in more acid solution, even native soy protein is gradually digested, though only at a rate of less than $\frac{1}{100}$ of that of denatured soy protein.[2] The gradual digestion of native soy inhibitor protein by pepsin at pH 2.0 is accompanied by a proportional loss in trypsin-inhibiting activity, so that no significant and definite change in the specific activity of the soy protein is brought about by treatment with pepsin.

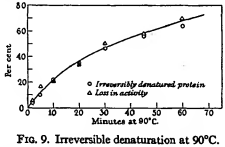

FIG. 9. Irreversible denaturation at 90°C.

Experimental Procedure.—(*a*) Digestion at pH 3.0 of denatured soy inhibitor: A tube containing 9 ml. of 0.25 per cent solution of soy inhibitor in 0.0025 M HCl was heated for 5 minutes at 90°C. and cooled for 2 minutes at 5°C. One ml. of 0.1 per cent solution of crystalline pepsin in 0.0025 M HCl was added and the mixture left at 30°C. Samples of 1 ml. were taken at various times and added to 5 ml. of boiling hot 5 per cent (0.3 M) trichloracetic acid. The precipitate was centrifuged after standing for about 1 hour at room temperature. The protein digest content of the clear supernatant was determined by measuring the optical density at 280 mμ. The results are given in Fig. 10, curve I.

(*b*) Digestion of native soy inhibitor at pH 3.0: Same procedure as in (*a*) except that the soy inhibitor solution had not been heated at 90°C. The results are given in Fig. 10, curve III. No digestion was observed during 6 hours.

(*c*) Digestion of soy inhibitor which was first denatured and then reversed: Same procedure as in (*a*), except that the soy solution after it had been heated to 90°C. was allowed to stand for 18 hours at 25°C. before the pepsin was added. See Fig. 10, curve II. The slight initial rise in curve II may be due to the presence of a small amount of irreversibly denatured protein, which was rapidly digested, the digestion then stopped.

[2] It is possible that at pH 2.0 or lower the protein becomes gradually denatured to a slight extent, and it is the denatured protein that is digestible by the pepsin.

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(*d*) Digestion at pH 2.0: Same procedure as in (*a*) and (*b*), except that the solution of soy inhibitor was in 0.01 M HCl. The results are shown in Fig. 11.

(*e*) Loss of inhibiting activity on digestion of soy protein by pepsin at pH 2.0: Same procedure as in (*b*) except that samples of the pepsin digestion mixture were also tested for trypsin-inhibiting activity, in addition to those tested for loss in protein.

The results given in Fig. 12 show that the loss in activity is parallel to the gradual digestion of the soy protein by pepsin.

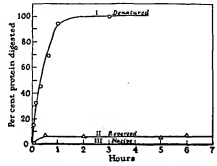

FIG. 10. Digestion of soy inhibitor by pepsin at pH 3.0.

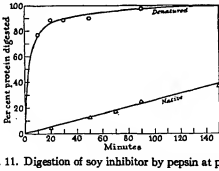

FIG. 11. Digestion of soy inhibitor by pepsin at pH 2.0.

2. Digestion of Soy Inhibitor Protein by Trypsin and Chymotrypsin.—Soy inhibitor, if denatured, is digestible by trypsin and chymotrypsin. However, in order to become susceptible to digestion by these enzymes the soybean protein has to be denatured more vigorously than when tested for pepsin digestion; the range of pH favorable for the action of trypsin and chymotrypsin is also favorable for the rapid reversal of denaturation of the soy inhibitor with the resulting inhibition of the proteolytic enzymes. It was found necessary to heat soy protein in 0.1 M NaOH for 10 minutes at 100°C. in order to make the protein susceptible to the digestive action of small amounts of trypsin or chymotrypsin. High concentrations of these enzymes undoubtedly digest soy protein even when less vigorously denatured. The measurement of digestion in the presence of relatively high concentrations of the enzymes is complicated by the autolysis of the enzymes, so that the measurements reflect

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not only the amount of substrates digested but also the digestion of the enzymes themselves.

Experimental Procedure.—Stock solution of 0.5 per cent of soy inhibitor in 0.1 M NaOH was heated for 10 minutes at 100°C., and cooled.

Digestion Mixture.—5 ml. of heated stock solution + 5 ml. 0.1 M HCl + 1 ml. 0.5 M phosphate buffer pH 7.4 + 1 ml. trypsin (0.2 mg.) in 0.0025 M HCl, or 1 ml. chymo-

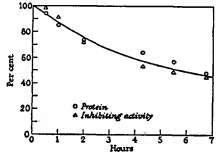

FIG. 12. Loss of trypsin-inhibiting activity of the soybean protein when digested by pepsin.

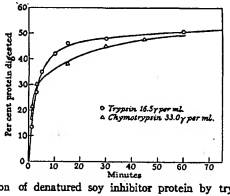

FIG. 13. Digestion of denatured soy inhibitor protein by trypsin and chymotrypsin.

trypsin (0.4 mg.). The mixture was left at 25°C. Samples of 1 ml. were mixed with 5 ml. of 5 per cent trichloracetic acid and centrifuged after standing several hours. Optical density of supernatant solutions was measured at 280 mμ. Corrections were made for the density reading of a blank in which a sample was mixed with trichloracetic acid before addition of trypsin or chymotrypsin. The results are shown in Fig. 13.

The rate of digestion was rapid in the initial stage of the reaction and slowed considerably at about 50 per cent digestion. The slowing of the digestion may be partly due to partial reversal of the substrate to native state at pH 7.6, enough to inhibit the proteolytic action of the enzymes, especially that of trypsin. It may also be due to a hydrolytic change brought about in the soy protein on heating in 0.1 M NaOH at 100°C., as evidenced by an increase in cor-

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rection for the blank (before addition of trypsin) over that of an unheated sample.

Chemical and Physical Properties of Soy Inhibitor

A summary of some of the chemical and physical properties of the soy inhibitor protein is given in Table VI.

TABLE VI

Chemical and Physical Properties of Crystalline Soybean Trypsin Inhibitor

Elementary analysis in per cent dry weight*	C..... 51.95 H..... 7.16 N..... 16.74 S..... 0.97 P..... 0.00 Ash..... 0.10
Tyrosine, *per cent dry weight*‡	4.0
Tryptophane, *per cent dry weight*§	2.2
Free amino nitrogen, *per cent total N*‖	4.0
Total Cu-phenol reagent color value, *mg. tyrosine equivalents per mg. protein*¶	0.21
Optical rotation $[\alpha]_D^{30}$ per gm. protein per ml. at pH 3.0	−105.0
Extinction coefficient at 280 mμ and at pH 3.0. Density per mg. protein per ml	0.91
Isoelectric point	pH 4.5
Molecular weight, by osmotic pressure measurement**	24000 ± 3000
Diffusion coefficient‡‡	0.07 − 0.08 cm.² per day at 24°C.

* Analysis carried out by Dr. A. Elek of The Rockefeller Institute, New York.

‡ Kindly determined by Miss Jean Grantham in the laboratory of Dr. E. Brand in the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, following NaOH hydrolysis by the method of Brand, E., and Kassell, B., *J. Biol. Chem.*, 1939, **131**, 489.

§ Colorimetric method of R. W. Bates (4). 1 ml. soy inhibitor containing about 5.0 mg. protein + 0.2 ml. 2.5 per cent $NaNO_2$ in H_2O + 0.5 ml. 5.0 per cent *p*-dimethyl-aminobenzaldehyde in 10 per cent H_2SO_4 + 15 ml. concentrated HCl.

Mixture left for 15 minutes in room then made up to 50 ml. with 50 per cent alcohol. Color was compared with that of 2.5 mg. of chymotrypsinogen treated in the same manner. The tryptophane content was calculated on the basis of 0.055 mg. tryptophane per milligram of chymotrypsinogen as determined by Brand and Kassell (5).

‖ Determined by formol titration (6).

¶ Method of Herriot (7). 1 ml. containing approximately 1 mg. protein + 0.0025 M $CuSO_4$ + 8 ml. 0.5 M NaOH + 3 ml. dilute Folin-Ciocalteau's phenol reagent (3) (1 part + 2 parts of water). The reagent is added drop by drop at a rapid rate. The color developed is compared with a similar mixture containing 0.205 mg. of tyrosine. The undiluted Folin-Ciocalteau's reagent is supplied by Hartman-Leddon Co., Philadelphia.

** Method of Northrop and Kunitz (8). Measurements in 0.5 M and 1 M NaCl pH 4.5 also in 0.5 M $MgSO_4$ pH 4.8.

‡‡ Method of Northrop and Anson (9).

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Methods

1. Estimation of Trypsin and Trypsin-Inhibitor Activities.—

Trypsin activity was measured either by the method of formol titration of gelatin or by digestion of casein.

(*a*) *Gelatin-Formol Method.*—The method is essentially the same as described before (10).

Digestion Mixture.—1 ml. of trypsin solution containing 0.01 to 0.05 mg. is mixed in a 50 ml. pyrex tube with 5 ml. of 5 per cent gelatin dissolved in 0.1 M phosphate buffer pH 7.6 and left at 35°C. for 20 minutes. The following reagents are then added in this order:

1 ml. formaldehyde, Merck Reagent
0.5 ml. 0.1 per cent phenolphthalein in 95 per cent alcohol
2 ml. 0.1 M NaOH

The mixture is titrated with 0.02 M HCl to the color of a standard.

The Color Standard.—

5 ml. 5 per cent gelatin
1 ml. formaldehyde
3 ml. H_2O
1 drop of 0.1 per cent phenolphthalein
Several drops of 1 M NaOH to maximum pink color

A blank is prepared in the same way as the "digestion mixture" except that the formaldehyde is added to the gelatin before addition of trypsin.

It is preferable to adjust the pH of the stock of 5 per cent solution of gelatin with 5 M NaOH so that the blank should require a titration of about 3 to 4 ml. of 0.02 M HCl. The range of concentration of trypsin used is such that the highest concentration of trypsin requires a titration of 0.5 to 1 ml. 0.02 M HCl.

The acid formed in the digestion mixture equals the blank titration value minus the titration value for the digestion mixture.

The method of calculation of tryptic activity is the same as that described for the casein method. One $[T.U.]^{Gel.\ F.} = 1$ milliequivalent acid formed per minute in the 6 ml. digestion mixture.

(*b*) *Casein Digestion Method.*—A stock solution of casein is made by suspending 1 gm. of casein (preferably "Hammarsten") in 100 ml. 0.1 M Sorensen's phosphate buffer pH 7.6. The suspension is heated for 15 minutes in boiling water, thus bringing about complete solution of the casein. The solution, designated as 1 per cent casein, is stored in the refrigerator and is stable for about a week or longer. Samples of 1 ml. of 1 per cent casein are pipetted into 15 ml. pyrex test tubes and placed in a water bath at 35°C. for about 5 minutes before being used.

The Trypsin Standard Curve.—One ml. samples of crystalline trypsin dissolved in 0.0025 M HCl or in a suitable buffer solution are added to samples of 1 ml. casein at intervals of about 1 minute, mixed well, and left at 35°C. for 20 minutes. The solutions are then poured back and forth into tubes containing 3 ml. of 5 per cent trichloracetic acid. The precipitates formed are centrifuged after standing 1 hour or longer at about 25°C. The concentration of split products in the supernatant solutions is determined either by the Cu-phenol reagent method as described in footnote ¶ (Table

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VI) on page 305 of this paper or by measuring the optical density of the solutions at 280 mμ. The optical density method is simpler and has been used throughout the present studies. The readings are corrected for blank solutions which are prepared by mixing 1 ml. of 1 per cent casein solution with 3 ml. of 5 per cent trichloracetic acid and then adding 1 ml. of the highest concentration of trypsin tested or 1 ml. of the buffer solution used in making up the trypsin dilutions. The corrections for blanks for the intermediate concentrations of trypsin are calculated by interpolation. The readings (corrected for blanks) are plotted as shown in Fig. 14. The plotted curve can be used for the determination of tryptic activity of any sample of material by reading the milligrams of trypsin corresponding to the corrected optical density reading of the sample. The activity is then expressed in terms of the sample of crystalline trypsin used for drawing the standard curve. A more general way is to calculate the specific activity of the trypsin and then replot the curve in terms of density *vs.* tryptic units.

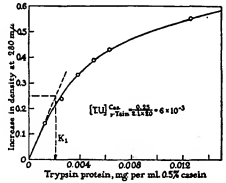

FIG. 14. Standard curve for digestion of casein by trypsin. Optical density at 280 mμ plotted *vs.* milligrams of trypsin protein.

The tryptic unit is defined as the activity which gives rise, under the conditions described, to an increase of one unit of optical density at 280 mμ per minute digestion, and is designated as $[T.U.]^{Cas.}$. The specific activity of the sample of trypsin used is obtained by drawing a straight line tangent to the first part of the curve. In Fig. 14, the slope $\frac{0.25}{2.1\gamma}$ (indicated by the dotted lines) divided by 20 minutes is the specific activity of the given material, *i.e.* the activity per microgram trypsin protein, *i.e.* $[T.U.]^{Cas.} = \frac{0.25}{2.1 \times 20} = 6 \times 10^{-3}$.

A new curve is then plotted (Fig. 15), the ordinates of which are identical with those in Fig. 14, while the abscissae are expressed in tryptic units, one γ being equal to 6×10^{-3} $[T.U.]^{Cas.}$. The data for Fig. 15 are conveniently obtained by reading the densities corresponding to 1γ, 2γ, 4γ, 6γ, etc., off the smooth curve of Fig. 14 and then plotting these values as ordinates against 6, 12, 24, 36×10^{-3} [T.U.] as abscissae. The data on the new curve are independent of the purity of the sample of trypsin used and

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hence it can be employed as a general standard curve for determination of tryptic activity, provided the same stock of casein is used and under the same experimental conditions of pH, temperature, etc.

The proteolytic activity of chymotrypsin is determined in the same way as that of trypsin.

(*c*) *Trypsin Inhibitor Activity Measurements.*—Inhibitor activity is expressed in terms of units of trypsin inhibited, and the measurement consists simply in comparing the tryptic activity of two samples of trypsin, one containing a definite amount of inhibitor and the other sample being free of inhibitor. The difference in the tryptic activity of the two samples of trypsin, provided the inhibitor is not in excess, expressed

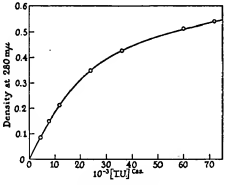

FIG. 15. Standard curve for digestion of casein by trypsin. Optical density at 280 mμ plotted *vs.* tryptic units.

in [T.U.] or in weight of pure trypsin divided by the weight of the inhibitor used is a measure of its specific activity.

Experimental Procedure.—Samples of 1 ml. containing 50 γ trypsin dissolved in 0.0025 M HCl were mixed with 1 ml. containing various amounts of soy inhibitor dissolved in 0.0025 M HCl. 1 ml. of each mixture added to 1 ml. of 1 per cent casein pH 7.6 was digested 20 minutes at 35°C., then mixed with 3 ml. 5 per cent trichloracetic acid and treated as described before. The measurements and the calculations are given in Table VII. The average specific activity of the inhibitor is about 1.0 when expressed in terms of weight of pure trypsin inhibited.

Crystalline soybean inhibitor because of its stability and purity can be used as a convenient standard for assaying samples of trypsin. The reaction with trypsin is independent of the method used for measuring the proteolytic activity of trypsin.

2. Protein Determination.—

(*a*) *Total N by Kjeldahl.*—The protein concentrations used in this paper were based on the total nitrogen determined by a semi-micro Kjeldahl method, 1 mg. of nitrogen being equivalent to 6.0 mg. of soybean protein.

Digestion Mixture.—1 ml. sample containing 2 to 5 mg. protein + 1 ml. concentrated H_2SO_4 + 1 drop selenium oxychloride + 0.25 gm. K_2SO_4 + several alundum chips. Digested 5 to 10 minutes in 100 ml. pyrex Kjeldahl flask, cooled, and 5 ml. H_2O added,

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then steam distilled in a ground-glass-jointed outfit, in the presence of 5 ml. 30 per cent NaOH.

The distillate is received in a flask containing 5 ml. 0.02 M HCl and is titrated with 0.02 M NaOH from a burette graduated to 0.01 ml. using methyl red as indicator.

(*b*) *Colorimetric Method by Means of Cu-Phenol Reagent According to Herriott as Described in Footnote* ¶ (*Table VI*), *page 305.*—The color developed is measured in a colorimeter or spectrophotometer at 600 mμ. The protein concentration is read on a standard curve obtained by plotting colorimeter or density readings *vs.* known concentrations of protein as determined by the Kjeldahl N method.

(*c*) *Optical Density Measurement at 280 mμ.*—A very convenient way of estimating protein in clear solutions is by measuring the ultraviolet light absorption at 280 mμ. The density readings are proportional to the concentration of protein up to density

TABLE VII

Trypsin Inhibiting Activity of Soy Inhibitor

Soy inhibitor per ml. 0.5 per cent casein, γ	0	2.5	5.0	7.5	10.0	12.5
Optical density at 280 mμ (corrected for blank)	0.550	0.515	0.455	0.348	0.185	0.008
10^{-6} [T.U.]$^{Cas.}$ read on curve, Fig. 15	75.5	61.5	43.7	25.5	10.5	0
10^{-6} [T.U.]$^{Cas.}$ inhibited (by difference)	0	14.0	31.8	50.0	65.0	75.5
Specific activity 10^{-6} [T.U.]$^{Cas.}$ per γ inhibitor		5.6	6.4	6.7	6.5	6.0
Average	1 γ inhibitor ≡ 6.2 × 10^{-6} [T.U.]$^{Cas.}$ ≡ 1.03 γ trypsin					

readings of almost 1.0. The proportionality constant varies, however, with different proteins.

The factors for calculating protein concentration from density measurement at 280 mμ are:

Soy bean inhibitor 1.10
Crystalline trypsin 0.585
Crystalline chymotrypsin 0.500.

The writer has been assisted in this work by Miss Barbara Brodsky.

SUMMARY

A study has been made of the general properties of crystalline soybean trypsin inhibitor. The soy inhibitor is a stable protein of the globulin type of a molecular weight of about 24,000. Its isoelectric point is at pH 4.5. It inhibits the proteolytic action approximately of an equal weight of crystalline trypsin by combining with trypsin to form a stable compound. Chymotrypsin is only slightly inhibited by soy inhibitor. The reaction between chymotryp-

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sin and the soy inhibitor consists in the formation of a reversibly dissociable compound.

The inhibitor has no effect on pepsin.

The inhibiting action of the soybean inhibitor is associated with the native state of the protein molecule. Denaturation of the soy protein by heat or acid or alkali brings about a proportional decrease in its inhibiting action on trypsin. Reversal of denaturation results in a proportional gain in the inhibiting activity.

Crystalline soy protein when denatured is readily digestible by pepsin, and less readily by chymotrypsin and by trypsin.

Methods are given for measuring trypsin and inhibitor activity and also protein concentration with the aid of spectrophotometric density measurements at 280 mμ.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Miri Seiberg, et al.
Serial No. 09/206,249'
Filed: December 7, 1998
Art Unit: 1654
Examiner: M. Meller
Attorney Docket No.: JBP 438

METHODS FOR REGULATING PHAGOCYTOSIS AND ICAM-1 EXPRESSION

DECLARATION OF MIRI SEIBERG, PH.D.

I, Miri Seiberg, am a Principal Research Fellow in the Skin Biology Technical Resource Center at Johnson & Johnson Consumer Companies, Inc. My education includes a Ph.D. in Molecular Biology from The Weizmann Institute of Science, Rehovot, Israel, in collaboration with Princeton University, Princeton, NJ and a B. S. in Life Sciences from Tel-Aviv University, Tel-Aviv, Israel. My curriculum vitae is attached hereto as Exhibit 1.

1. Soybeans are of high nutritional value, however they have been known to possess certain undesirable qualities which limit their use in animal and human nutrition. (e.g. Bau HM , Alais C Denaturation and enzymatic proteolysis in vitro of protein fractions of soya flour Ann Nutr Aliment. 1975;29(4):351-70). Numerous studies have evaluated the effects of technological treatments on the properties of certain soybean protein fractions, in order to identify a fraction with nutritional value but no gastro-intestinal side effects. (Bau HM , Alais C Denaturation and enzymatic proteolysis in vitro of protein fractions of soya flour Ann Nutr Aliment. 1975;29(4):351-70)

2. Soy products containing soy trypsin inhibitor activity are and were well-known to be dangerous for human ingestion. Soy products containing such activity can cause gastric distress and even death due to non-absorption of nutrients. In fact, the World Health Organization has issued warnings about ingesting uncooked legume

products for this very reason. Thus, any publications that indicate that a soy or legume product is ingestible imply that such a product would not have contained active trypsin inhibitors.

3. Proteins are said to be "denatured" when their physical and physiological properties are changed such that they lose their activity. Such change is generally due to a change in a protein's chemical structure and/or conformation. Protein denaturation and the consequent loss of biological activity are described in biochemistry textbooks (e.g. Biochemistry, A. L. Lehninger, 1975, p.62-63).

4. The soy products of the compositions of my invention must contain active trypsin inhibitors in order to be effective in decreasing phagocytosis or ICAM-1 induction. I understand this to mean that they are non-denatured, and cannot be used for nutritional consumption.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dr. Miri Seiberg

1/17/06
Date

Exhibit 1

Miri Seiberg
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Education

1977 B.Sc. Biological Sciences, Tel-Aviv University, Israel.
1982 M.Sc. Biochemistry, The Weizmann Institute of Science, Israel.
1989 Ph.D. Molecular Biology, The Weizmann Institute of Science, Israel, in collaboration with Princeton University, Princeton NJ.

Employment

1982 **The Weizmann Institute of Science, Israel.**
Research assistant, Dept. of Chemical Immunology.

1982-90 **Princeton University, Princeton NJ**
1982-84 Visitor, Dept. of Biochemical Sciences.
1987-89 Visitor, Dept. of Molecular Biology.
1989-90 Post Doctoral Fellow, Dept. of Biology.

1990- 92 **Bristol-Myers Squibb PRI, Princeton NJ.**
Post Doctoral Fellow, Dept. of Macromolecular Structure.

1992- **Johnson & Johnson Family of Companies**
1992-95 Senior Scientist, Skin Biology Research Center of Pharmaceutical Research Institute, Raritan NJ.
1995-96 Staff Scientist, Dermatology R&D, Johnson & Johnson Consumer Companies, CPWW division, Skillman NJ.
1997-99 Principal Scientist, Skin Research Center, CPWW, Skillman NJ
1999-0 Research fellow, Skin Research Center, CPWW, Skillman NJ
2001 -05 Sr. Research fellow, Skin Biology TRC and LAS, CPWW, Skillman NJ
5/2005- Principal Research fellow, Skin Biology TRC and LAS, CPWW, Skillman NJ

Industrial Experience

1990- 92, Bristol-Myers Squibb PRI, Princeton NJ.

Post Doctoral Fellow, Dept. of Macromolecular Structure.

Using a rat model system for salt-induced hypertension, identified a novel gene involved in salt-induced hypertension, and demonstrated selective expression patterns.

1992-today, Johnson & Johnson Pharmaceutical Research Division

1992-95, Senior Scientist

This position involves conducting individual projects, supervising one BS/MS technician. Identified pathways involved in epidermal differentiation, hair growth and keratinocyte apoptosis. Developed relevant bioassays and screens.

Johnson & Johnson Consumer Companies, Inc.

1995-96, Staff Scientist

Directed two research scientists. Developed enzymatic, molecular and cellular assays and screens for potential drug and cosmetic activity. Involved in retinoid studies, proteases and protease inhibitors, in epidermal differentiation and hair growth.

1997-99, Principal Scientist

Head of pigmentation group. Directed research scientists and postdoctoral fellows. Horizontally directed the pigmentation technology development team. Initiated and directed molecular, cellular, and biochemical studies of pigmentation, resulting in the identification of a novel pathway that regulates skin color. Identified agents, both drugs and cosmetics, to modulate this pathway, resulting in darkening or lightening of human skin. Designed and evaluating product prototypes for biological activity and efficacy. In charge of numerous academic collaborations.

1999-00, Research Fellow

Continue heading the pigmentation team and supporting technology and product design groups in creating a line of depigmenting agents. First products available in stores. Additional responsibility in heading the hair growth efforts, introducing a new concept for delaying hair growth. Identified novel cosmetic agents with modulatory effect, demonstrated preclinical POP and initiated product development efforts. Expand responsibility for academic collaborations.

2001 - 2005, Sr. Research Fellow

Director of the Skin Biology research group, including pigmentation, hair, acne, skin aging and skin cancer teams and supporting facilities. Continue basic research and product development support in all areas. Identified a novel cosmetic for skin aging, currently under early development stages. Directed efforts in the development of a new drug for acne, based on a proprietary target, now under clinical evaluation. Continue R&D support for skin lightening technology, now sold by numerous Brands and J&J companies worldwide. Continue R&D support for delaying hair growth technology, now sold by numerous J&J companies and Brands worldwide. Received the Johnson Medal, the highest level of scientific recognition by JJ. Head of Laboratory Animal Services, incl. vivarium support for numerous J&J companies. Council member of the J&J Corporate office of Science and Technology. In charge of academic interactions and collaborations for Skin Biology and related areas. In charge of the J&J SRC training grant. Member of the mentoring team.

5/2005, Principal Research Fellow

Patent applications

More than 25 patent applications in the areas of skin and hair

J&J Awards

1. Skin care council – best scientific content poster award. June 1993.
2. American Express achievement award of PRI. January 1995.
3. COSAT-CORD internship award. April 1997.
4. Skin care council – best overall poster award. June 1999.
5. COSAT excellence in science award. November 1999.
6. CPWW achievement award. January 2000.
7. Skin care council – best overall poster award. June 2001.
8. CPPW Grandview award. March 2003.
9. The Johnson Medal. Oct 2003.
10. The Mountainview award. March 2005.

Societies

1. Pan American Society of Pigment Cell Research (council member, 2001-03, member of finance committee, 2000-02, nominated for 2005 presidency elections).
2. Society of Investigative Dermatology
3. American Society of Cellular Biology
4. American Association for the Advancement of science
5. New York Academy of Science (elected 2003).

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Y. Aloni, O. Resnekov, **M. Selberg**, M. Kessler and A.J. Levine. CSH RNA processing meeting (1986), p. 23. Eucaryotic RNA polymerase II can prematurely terminate transcription at precise sites that resemble a procaryotic termination signal.

M. Selberg and Y. Aloni. CSH DNA Tumor virus meeting (1986), p. 128. Attenuation in Ad2 may determine its host-range.

Y. Aloni, M. Kessler, O. Resnekov, **M. Seiberg**, N. Ragimov, E. Bengal and O. Amster-Choder. CSH RNA processing meeting (1987), p. 21. Attenuation in the regulation of gene expression in virus and animal cells.

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M. Seiberg, C. Paine, E. Sharlow, P. Andrade-Gordon, M. Costanzo, M. Eisinger and S. Shapiro (1999). Pigment Cell Research Supp 7, p. 41. The Protease-Activated Receptor-2 regulates pigmentation via keratinocyte-melanocyte interactions. IPCC international meeting of pigmentation (Invited speaker).

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A. Deng, **M. Seiberg**, A. Pentland, R. Han and G. Scott (Oct 2000). The American Society of Dermatopathology 36th annual meeting. Protease-Activated Receptor-2 (PAR-2), a receptor involved in melanosome transport, is upregulated in skin in vivo by Ultraviolet irradiation.

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M. Seiberg, C. Paine, E. Sharlow, M. Eisinger and S. S. Shapiro (March 2001). 59th annual meeting of the American Academy of Dermatology. The PAR-2 pathway can regulate pigmentation.

C. Paine, E. Sharlow, F. Liebel, M. Eisinger, S. Shapiro and **M. Seiberg** (March 2001). 59th annual meeting of the American Academy of Dermatology. An alternative approach to depigmentation by Soybean extracts *via* inhibition of the PAR-2 pathway

M. Selberg, C. Paine, J-C Liu and S. Shapiro (March 2001). 59th annual meeting of the American Academy of Dermatology. Soymilk and soybean-derived proteins delay hair growth and reduce hair size and hair pigmentation

C. Paine, R. Gallaghar, and **M. Selberg** (March 2001). 59th annual meeting of the American Academy of Dermatology. Computerized image analysis for hair growth studies.

C. Paine, G. Payonk, and **M. Selberg** (March 2001). 59th annual meeting of the American Academy of Dermatology. Computerized image analysis for pigmentation studies.

M. Selberg, C. Paine, J-C Liu and S. Shapiro (March 2001). 59th annual meeting of the American Academy of Dermatology. Soymilk prevents UV-induced pigmentation and reduces sunburns.

J-C Liu, **M. Selberg**, S. Shapiro and R. Grossman (March 2001). 59th annual meeting of the American Academy of Dermatology. Soy: Potential applications in skin care.

C Paine, L. Babiarz, E. Sharlow, F. Liebel, M. Eisinger, SS Shapiro and **M. Selberg**., An Alternative Approach to Depigmentation by Soybean Extracts via Inhibition of the PAR-2 pathway. Society for Investigative Dermatology Annual Meeting (2001) abstract # 731 p.52.

M. Selberg, L. Babiarz, J.C. Liu, and S.S. Shapiro. Soymilk Reduces Hair Growth and Follicle Dimensions. Society for Investigative Dermatology Annual Meeting (2001) abstract # 261 p.36.

C. Paine, E. Sharlow, L. Babiarz, F. Liebel, M. Eisinger, S. Shapiro and **M. Selberg.** 10th congress of the European Academy of Dermatology and Venerology. An alternative approach to depigmentation by Soybean extracts *via* inhibition of the PAR-2 pathway (Oct 2001).

J.C. Liu, **M. Selberg**, J. Miller and J. Wu. 10th congress of the European Academy of Dermatology and Venerology. Application of Soy in skin care (Oct 2001).

M. Selberg, C. Paine, M. Eisinger and S. Shapiro. 10th congress of the European Academy of Dermatology and Venerology. The PAR-2 pathway can regulate pigmentation (Oct 2001).

J.C. Liu, **M. Selberg**, J. Miller, J. Wu, S. Shapiro and R. Grossman. 4th International Symposium on the role of Soy in preventing and treating chronic disease. Applications of soy in skin care. (2001).

J.C. Liu, **M. Selberg**, F. Liebel, T. Chen, Y. Appa, and T. Oddos. Pre-clinical and clinical evaluation of Total Soy preparations in improving skin physical tone parameters. 60th annual meeting of the American Academy of Dermatology. (Feb 2002).

C. B. Lin, L. Babiarz, F. Liebel, Roydon E. Price, D. E. Fisher, G. J. Gendimenico, and **M. Selberg.** Modulation of microphthalmia-associated transcription factor expression alters skin pigmentation. Society for Investigative Dermatology Annual Meeting (Apr. 2002). J. Invest. Dermatol 119, p.339

L. Babiarz, C. B. Lin and **M. Selberg.** Differential expression of PAR-2 and its activator trypsin in different skin types. Society for Investigative Dermatology Annual Meeting (Apr. 2002). J. Invest Dermatol. 119, p.340.

M. Selberg, L. Babiarz, J.C. Liu, and S.S. Shapiro. Soymilk Reduces Hair Growth and Follicle Dimensions. European Hair Research Society 9th meeting (June 2002).

C. B. Lin, L. Babiarz, F. Liebel, Roydon E. Price, D. E. Fisher, G. J. Gendimenico, and **M. Seiberg**. Modulation of microphthalmia-associated transcription factor expression alters skin pigmentation. International Pigment Cell Conference (Sept. 2002).

L. Babiarz, C. B. Lin and **M. Seiberg**. Differential expression of PAR-2 and its activator trypsin in different skin types. International Pigment Cell Conference (Sept. 2002).

M. Seiberg, M. Filippi, A. Ricklis, V. Iotsova, C. B. Lin. Zinc Pyrithione Delays Hair Growth and Follicular Melanogenesis. International Investigative Dermatology Annual Meeting (Apr. 2003, #784).

Glynis Scott, Sonya Leopardi, Lorelle Parker, Laura Babiarz, **Mirl Seiberg**, Rujiing Han. The PAR-2 receptor mediates phagocytosis in a Rho-dependent manner in human keratinocytes. International Investigative Dermatology Annual Meeting (Apr. 2003, #1146, Invited talk).

M. Seiberg, L. Babiarz, and C. B. Lin. Differential expression of PAR-2 and its activator trypsin in different skin types. European Pigment Cell Conference (Sept. 2003) (Invited speaker).

M. Seiberg, PAR-2 affects pigmentation via melanosome transfer. 2nd SkinEthic conference (Oct 03, Amsterdam, Nederland), (invited speaker).

M. Seiberg, Soy story, a case study, R&D Summit (Oct 03, Nice, France), (invited speaker).

M Huang, J Xie, CB Lin, M Kizoulis, **M Seiberg**, S Shapiro, and AH Conney. Inhibitory effect of topical applications of non-denatured soymilk on the formation and growth of UVB-induced skin tumors. 65th SID Annual Meeting (April 28-May 1, 2004). Poster Number 091, p.46.

L Babiarz-Magee, N Chen, M Kizoulis, **M Seiberg** and CB Lin. Expression and activation of protease-activated receptor-2 correlate with skin color. 65th SID Annual Meeting (April 28-May 1, 2004). Poster Number 888, p. 80.

Z Renbin, J-C Liu, C Bertin, JP Ortonne, **M Seiberg**, V Iotsova. Non-denatured soybean extracts enhance skin elasticity. 65th SID Annual Meeting (April 28-May 1, 2004). Poster Number 169, p. 49.

M. Seiberg. Melanosomes and PAR-2: From basic research to applications. PASPCR (June 2004) (invited speaker).

M Seiberg, CB Lin, MT Huang, JG Xie, M Kizoulis, AH Conney, and S Shapiro. The Inhibitory Effect of Topical Applications of Non-denatured Soy Extracts on UVB-induced Erythema, DNA Damage, and Apoptosis, and on the Formation and Growth of-Skin Tumors. PASPCR (June 2004).

M Seiberg, CB Lin, MT Huang, JG Xie, M Kizoulis, AH Conney, and S Shapiro. The Inhibitory Effect of Topical Applications of Non-denatured Soy Extracts on UVB-induced Erythema, DNA Damage, and Apoptosis, and on the Formation and Growth of-Skin Tumors. The 32nd Annual Meeting of the American Society for Photobiology (2004).

M Seiberg, CB Lin, MT Huang, JG Xie, M Kizoulis, AH Conney, and S Shapiro. The Inhibitory Effect of Topical Applications of Non-denatured Soy Extracts on UVB-induced Erythema, DNA Damage, and Apoptosis, and on the Formation and Growth of-Skin Tumors. The annual Montagna Symposium on the Biology of Skin (2004) (Invited talk).

V Iotsova, R Zhao, JC Liu, C Bertin, JP Ortonne, **M Seiberg**. Changes in elastin expression and organization in response to treatments with non-denatured soybean extracts. 2nd national meeting of the Amer. Soc for Matrix Biology (2004).

C. Gell, C. B. Lin, N. Chen, **M. Seiberg** and M. Harris. Reconstituted Buccal Tissue Integrity and Proinflammatory Cytokine Response of Commercial Mouthwashes. 83rd meeting of the International Association for Dental Research. (Oral presentation). J. of Dental Res vol. 84 (special issue A, abst. # 80, 2005).

M Seiberg, CB Lin, MT Huang, JG Xie, M Kizoulis, AH Conney, and S Shapiro. The Inhibitory Effect of Topical Applications of Non-denatured Soy Extracts on UVB-induced Erythema, DNA Damage, and Apoptosis, and on the Formation and Growth of-Skin Tumors. Keystone symposia (3/2005) Genome Instability and Repair (C2)

CB Lin, R Scarpa, N Chen, L Babiarz-Magee, M Kizoulis, S Shapiro and **M Seiberg**. Protease-activated receptor-2 activating peptides mediate skin pigmentation and distinctly different signal transduction. 66th SID Annual Meeting (2005). Poster Number 841.

N Chen, **M Seiberg** and CB Lin. Cathepsin L2 expression levels inversely correlate with skin color. 66th SID Annual Meeting (2005). Poster Number 843.

CB Lin, R Scarpa, N Chen, L Babiarz-Magee, M Kizoulis, S Shapiro and **M Seiberg**. Protease-activated receptor-2 activating peptides mediate skin pigmentation and distinctly different signal transduction. IPCC Meeting (2005).

N Chen, **M Seiberg** and CB Lin. Cathepsin L2 expression levels inversely correlate with skin color. IPCC Meeting (2005).

N Chen, M Seiberg and CB Lin. Cathepsin L2 expression levels inversely correlate with skin color. IPCC Meeting (2005). 3rd International Symposium on Ethnic Hair and Skin: Advancing the Scientific Frontier.

JAN 16 2004 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Miri Seiberg, et al.
Serial No. **09/206,249**
Art Unit: **1651**
Examiner: **M. Meller**
Attorney Docket No.: **JBP 438**

METHOD FOR REGULATING PHAGOCYTOSIS

DECLARATION OF ROBERT ZIVIN, PH.D.

I, Robert Zivin, am a Corporate Director in the Corporate Office of Science and Technology at Johnson & Johnson. My education includes a Ph.D. in Microbiology from University of Chicago and a B. S. in Biology from the Northern Illinois University. My curriculum vitae is attached hereto as Exhibit 1.

1. This Declaration is respectfully submitted to discuss the process of denaturation of proteins. Proteins are defined by both (1) their chemical structure, which includes its substituent amino acids as well as their unique conformation and (2) their biological function. Only when a protein has its unique chemical structure and conformation does it exhibit its biological function or activity.

2. Proteins are made by joining amino acids by a peptide bond, which is formed in a reaction between the amino group of one amino acid and the carboxyl group of another amino acid. (Messier, P., "Protein Chemistry of Albumen Photographs", *Topics in Photographic Preservation*, Vol. 4, 1991, pp. 124-135, http://albumen.stanford.edu).

3. Proteins have a particular conformation that is characteristic of each specific type. This conformation forms as a result of the interactions of the protein's

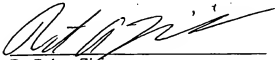
component amino acids as they seek the lowest free energy state for the protein. (Messier, P., 1991). "Only when in its native, or low energy, state does a protein manifest all of its characteristic properties and biological functions..." (Messier, P., 1991). Thus, conformation is an important aspect of protein structure and identification.

4. "Protein denaturation" is defined as "the process of altering the native/low free energy conformation of a protein." (Messier, P., 1991). Once the protein is denatured, by exposure to increased temperature, its characteristic activity is no longer present and it cannot be detected in a composition. Denaturation generally only alters the conformation of a protein and does not chemically break the peptide bonds. In theory, this process may be reversible with some proteins.

5. However, in the case of most proteins, including those proteins contained in soy exhibiting Soy Trypsin Inhibition ("STI") activity, denaturation is irreversible. Soy proteins are soluble proteins—they are folded such that their hydrophobic substituents are internal to the conformation. Denaturation opens up the conformation, exposing the inner hydrophobic substituents. This often causes the denatured proteins to precipitate out of solution, removing them from the composition in which they reside. However, in any case, the elimination of biological activity is independent of the presence or absence of the denatured protein.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title

18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Dr. Robert Zivin

12/23/03

Date

Exhibit 1

Robert Allan Zivin, Ph.D.
CURRICULUM VITAE,
September 2003

ADDRESS: 9 Pebble Beach Ct. PHONE: 609-333-0153
Skillman, NJ 08558 CELL: 609-933-6719

EDUCATION:

University of Chicago, Chicago, IL Ph.D. 1980 Microbiology
Northern Illinois University, Dekalb, IL B.S. 1975 Biology

PROFESSIONAL ORGANIZATIONS:

Society for Biomolecular Screening
AAAS

ACADEMIC APPOINTMENTS:

Adjunct Professor, Department of Chemical & Biochemical Engineering, Rutgers University, Piscataway, NJ 1995-present

OUTSIDE ACTIVITIES

Program Committee, Photonics West-BIOS (SPIE), 1999 & 2000
Member, Board of Directors, Joint Biotechnology Training Grant, Rutgers U / UMDNJ
External Reviewer, New Jersey Commission on Science & Technology, 1999-2003
Organizing Committee & Session Chairman, IBC ScreenTech 2001-2004, Protein Kinases 2002

EMPLOYMENT:

1986 -	Johnson & Johnson
2003-Technology	Corporate Director, Johnson & Johnson Office on Science and
2002-2003	Team Leader & Research Fellow, J&J PRD
1999 -2002	Team Leader & Research Fellow, R. W. Johnson PRI
1995 -1998	Team Leader, Exploratory Technology Team, R. W. Johnson PRI
1993-1995	Principal Scientist, Growth Factors Team, R. W. Johnson PRI
1990-1993	Principal Scientist, PRI Biotech, R. W. Johnson PRI
1989-1990	Senior Scientist, PRI Biotech, R. W. Johnson PRI
1986-1989	Ortho Diagnostic Systems, Inc.

Senior Scientist, DNA Probe Diagnostics

1985 - 1986 Cistron Technology Inc., Pine Brook, NJ
Senior Scientist

1983 - 1985 Merck & Co.
West Point, PA
Senior Research Microbiologist

1980 - 1982 National Cancer Institute-Frederick Cancer Research Center
Frederick, MD - Postdoctoral Fellow

PUBLICATIONS

Blank, K.; Mai, T.; Gilbert, I.; Schiffmann, I.; Rankl, J.; Zivin, R.; Tackney, C.; Nicolaus, T., Spinnler, K.; Oesterhelt, F.; Benoit, M.; Clausen-Schaumann, H. and Gaub, H.E. A force-based protein biochip. Proc. Nat. Acad. Sci. USA (2003) 100: 11356-11360

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Robert A. Fluorescence Imaging of Electrically Stimulated Cells. Journal of Biomolecular Screening, in press (December 2003).

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PATENTS

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Awards

J&J Achievement Award, 1996. Small molecule EPO mimetics.

PRI Discovery Award, 1991. Humanization of monoclonal antibodies.

DRAFT – 12/11/2001

Docket No. JBP 438

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Seiberg, et al.	
Serial No.	:	09/206,249	Art Unit: 1651
Filed	:	December 7, 1998	Examiner: M. Meller
For	:	METHOD FOR REGULATING PHAGOCYTOSIS	

#23
A.Q.J
4/24/03

DECLARATION OF KATHARINE MARTIN

I, Katharine Martin, hereby declare:

1. I am currently employed by Johnson & Johnson Consumer Companies, Inc. in the capacity of Manager, Pharmacology. I began employment with Johnson & Johnson Consumer Products, Inc. in 1990 as Scientist, *In Vitro* Toxicology.

2. I received a Bachelors of Science degree from the University of Bath in England. I am knowledgeable in the area of protein activity due to my experience in Biological Sciences.

3. Prior to 1986, it was well-known to those skilled in the art of protein biochemistry that the conformation of proteins, particularly their native tertiary and quaternary structure are important for such proteins' activity. The term "activity" is defined as a physiological process or participation in a biochemical reaction, e.g. the ability of an enzyme to cause a modification of substrate. In order to be active, proteins should retain their native structure. Proteins will not be active once they are subjected to forces that tend to disrupt their native structure physically or chemically and, thereby, denature them. Forces that can denature proteins include, but are not limited to, pH changes, detergents and excessive heating. (See, e.g., Biological sciences, 4th edition, Keton and Gould, eds., chapter 3, e.g. p.67, 1986).

4. Soybeans were first cultivated in Asia as a crop rotation material (circa 1134-246 BC) (Soybeans, Chemistry, Technology and Utilization, Edited by K. Liu, page 1-3, history, 1999.). During this time, soybeans were not consumed as food due to serious gastric distress that resulted from eating the raw bean (Soybeans, Chemistry, Technology and Utilization, Edited by K. Liu, page 1-3, history, 1999.). Once precipitation and fermentation techniques were developed, Soybeans were incorporated into the Chinese diet. Heat

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inactivation of proteins present in the soybean such as Soybean Trypsin Inhibitor ("STI") and Bowman-Birk Inhibitor ("BBI") during soybean processing renders soybeans edible (reviewed in (Wallace et al., 1971), (Kwok and Niranjan, 1995). It was known that the observed gastric distress is the result of inhibition of protein digestion by trypsin and other digestive proteases by the potent serine protease inhibitors, STI and BBI.

5. The negative effects of native STI to the digestive system are heavily documented. Silva et al (1986) documented morphological alterations of small intestinal epithelium, caused by feeding calves with non-denatured soy proteins. Pancreatic enlargement induced by orally ingested STI was documented and studies by Wilson et al (1978) and Krogdahl et al (1979) and reviewed by Flavin (1982). Liener (1983) summarized similar observations in a publication entitled "Naturally occurring toxicants in foods and their significance in the human diet".

6. These studies and others state that STI should be inactivated when soybeans are processed for nutritional use. Numerous studies were conducted to evaluate the effect of processing conditions on the trypsin inhibitory activity and digestibility of various soy preparations (e.g. Wallace et al, 1971, reviewed in Kwok et al, 1995). Today, soy products marketed for nutritional use are processed (e.g. pasteurized, fermented, or cooked) in order to inactivate STI (see e.g. the book: Soybeans, chemistry, technology and utilization, K. Liu, Ed, 1999).

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: February 8th 2002

K. M. Martin

Katharine Martin

EXHIBIT C

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Miri Seiberg, et al.
Serial No. 09/206,249
Art Unit: 1651
Examiner: D. M. Naff
Attorney Docket No.: JBP 438

METHOD FOR REGULATING PHAGOCYTOSIS

DECLARATION OF MIRI SEIBERG AND STANLEY S. SHAPIRO UNDER 37 C.F.R. § 1.132

I, Miri Seiberg, do hereby declare that I am a Senior Research Fellow for the applicant of the above-identified patent application. I, Stanley S. Shapiro, do hereby declare that I am Vice President of the Corporate Office of Science and Technology for the applicant of the above-identified patent application.

1. We were two (2) of the inventors of Application No. 09/110,409 ("'409 Application").
2. We invented the invention claimed in claims 24, 28-36, 38, 40, 44-47 and 58 of the present application, Application No. 09/206,249.
3. The disclosed but not claimed invention of the '409 Application, which is covered by claims 24, 28-36, 38, 40, 44-47 and 58 of the present application was our invention, and not the invention of another.

We each further declare that all statements made herein of our own individual knowledge are true and that all statements made on information and belief are believed to be true,

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and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application and any registration resulting therefrom.

Date:_____

Miri Seiberg

Date: 4/21/03

Stanley S. Shapiro

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Miri Seiberg, et al.
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1. We were two (2) of the inventors of Application No. 09/110,409 ("'409 Application").
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and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application and any registration resulting therefrom.

Date: 4/22/03 Mirl Seiberg

Date: Stanley S. Shapiro

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EXHIBIT A

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23. (cancelled)

24. A method of decreasing phagocytosis or ICAM-1 expression in a mammalian cell in need thereof, comprising contacting the cell with a therapeutically phagocytosis- or ICAM-1 decreasing effective amount of soybean milk containing soybean trypsin inhibitor that specifically decreases phagocytosis or ICAM-1 expression.

25. (cancelled)

28. The method of claim 24, wherein the agent inhibits the PAR-2 pathway.

29. The method of claim 24, wherein the agent is selected from the group consisting of a soybean derivative and a serine protease inhibitor.

30. The method of claim 29, wherein the agent is selected from the group consisting of soybean milk containing soybean trypsin inhibitor.

31. The method of claim 23 or 24, wherein the mammalian cell is a PAR-2-expressing cell.

32. The method of claim 31, wherein the mammalian cell is selected from the group consisting of a keratinocyte, a fibroblast, and a professional phagocyte.

33. The method of claim 32, wherein the mammalian cell is a keratinocyte.

34. The method of claim 32, wherein the mammalian cell is a fibroblast.

35. The method of claim 32, wherein the mammalian cell is a professional phagocyte.

36. The method of claim 23 or 24, wherein the mammalian cell is a human cell.

37. (cancelled)

38. A method of treating a mammal afflicted with a disorder ameliorated by a decrease in phagocytosis or ICAM-1 expression in appropriate cells, which comprises topically administered to the cells in need thereof a phagocytosis- or ICAM-1-decreasing therapeutically effective

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amount of soybean milk containing soybean trypsin inhibitor that specifically decreases phagocytosis or ICAM-1 expression.

39. (cancelled)

40. A method of preventing a mammal afflicted with a disorder ameliorated by a decrease in phagocytosis or ICAM-1 expression in appropriate cells, which comprises topically administering to the cells in need thereof a prophylatically phagocytosis- or ICAM-1 decreasing effective amount of soybean milk containing soybean trypsin inhibitor that specifically decreases phagocytosis or ICAM-1 expression.

41. (cancelled)

44. The method of claim 38 or 40, wherein the agent inhibits the PAR-2 pathway.

45. The method of claim 38 or 40, wherein the agent is selected from the group consisting of soybean milk containing soybean trypsin.

46. The method of claim 45, wherein the agent is selected from the group consisting of soybean milk containing soybean trypsin inhibitor.

47. The method of claim 37, 38, 39 or 40, wherein the appropriate cells are PAR-2-expressing cells.

48. (cancelled)

49. (cancelled)

50. (cancelled)

51. (cancelled)

52. (cancelled)

53. (cancelled)

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54. (cancelled)

55. (cancelled)

56. (cancelled)

57. (cancelled)

58. The method of claim 37, 38, 39 or 40, wherein the mammal is a human.

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EXHIBIT A

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 27, Issue of July 14, pp. 16645–16652, 1995
Printed in U.S.A.
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Structural and Functional Roles of Asparagin 175 in the Cysteine Protease Papain*

(Received for publication, January 5, 1995, and in revised form, May 10, 1995)

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The role of the asparagine residue in the Cys-His-Asn "catalytic triad" of cysteine proteases has been investigated by replacing Asn^{175} in papain by alanine and glutamine using site-directed mutagenesis. The mutants were expressed in yeast and kinetic parameters determined against the substrate carbobenzoxy-L-phenylalanyl-(7-amino-4-methylcoumarinyl)-L-arginine. At the optimal pH of 6.5, the specificity constant $(k_{cat}/K_m)^{obs}$ was reduced by factors of 3.4 and 150 for the $Asn^{175} \rightarrow Gln$ and $Asn^{175} \rightarrow Ala$ mutants, respectively. Most of this effect was the result of a decrease in k_{cat}, as neither mutation significantly affected K_m. Substrate hydrolysis by these mutants is still much faster than the non-catalytic rate, and therefore Asn^{175} cannot be considered as an essential catalytic residue in the cysteine protease papain. Detailed analyses of the pH activity profiles for both mutants allow the evaluation of the role of the Asn^{175} side chain on the stability of the active site ion pair and on the intrinsic activity of the enzyme. Alteration of the side chain at position 175 was also found to increase aggregation and proteolytic susceptibility of the proenzyme and to affect the thermal stability of the mature enzyme, reflecting a contribution of the asparagine residue to the structural integrity of papain. The strict conservation of Asn^{175} in cysteine proteases might therefore result from a combination of functional and structural constraints.

Cysteine proteases are a class of enzymes requiring the thiol group of a cysteine residue for their catalytic activity (1). The additional involvement of an histidine residue in the catalytic process was inferred on kinetic grounds (2), and evidence for the location of an histidine in proximity to the catalytic thiol group was provided initially by the use of a bifunctional irreversible inhibitor of papain (3). The Cys^{25}-His^{159} arrangement in the catalytic center of papain was established when the three-dimensional structure of the enzyme was solved (4–5). The papain molecule is folded to form two interacting domains delimiting a cleft at the surface of the enzyme. Cys^{25} and His^{159} are located at the interface of this cleft on opposite domains of the molecule; Cys^{25} is part of the L1 α-helix at the surface of the left domain, while His^{159} is in a β-sheet at the surface of the right domain of the enzyme.

With the availability of the three-dimensional structure, other residues were found in the vicinity of the active site that could possibly play important roles in the mechanism of the enzyme. In particular, an asparagine residue that is conserved in all cysteine protease sequences of the papain family, Asn^{175}, was found to be adjacent to the catalytic His^{159} residue. The amide oxygen of the Asn^{175} side chain is hydrogen-bonded to $N^{\delta}H$ of His^{159} creating a Cys-His-Asn triad, which can be considered as being analogous with the Ser-His-Asp triad of serine proteases (Fig. 1). The side chain of Asn^{175} is buried in a hydrophobic region of the enzyme composed mainly of residues Phe^{141}, Val^{151}, Trp^{177}, and Trp^{183}. Residues 141, 177, and 181 are located near the Asn^{175}-His^{159} hydrogen bond and can shield it from the external solvent. An important feature of the Asn^{175}-His^{159} interaction is that the hydrogen bond is approximately coplanar with the His^{159} C^{δ}-C^{γ} bond, allowing rotation of the imidazole ring about the C^{β}-C^{γ} bond without disruption of the Asn^{175}-His^{159} hydrogen bond. Comparison of results from crystallographic studies with various forms of papain either free or alkylated at the Cys^{25} sulfur atom by chloromethyl ketone inhibitors have demonstrated that the His^{159} side chain can change its orientation by about 30° (7). Therefore, it has been suggested that the role of Asn^{175} is to orient the His^{159} side chain in the optimum positions for various steps of the catalytic mechanism. In the resting state of the enzyme, the His side chain would be coplanar to the Cys^{25} residue while during acylation, the protonated imidazole ring would rotate to act as a proton donor to the nitrogen atom of the leaving group of the substrate (8).

An important feature of papain and other cysteine proteases in general is the high nucleophilicity of the sulfur atom of the active site cysteine residue. This is due to the fact that at the pH values where the enzyme is active, the sulfur atom is present as a thiolate anion. It is now generally accepted that the side chains of Cys^{25} and His^{159} possess unusual pK_a values and that the active form of the enzyme consists of a thiolate-imidazolium ion pair at neutral pH (9–12). However, the nature and significance of the factors that are responsible for the formation and maintenance of the ion pair within a wide range of pH for the most part remain unknown, and this aspect has been the object of many theoretical studies over the years (see, e.g., Refs. 13–19). Since the side chains of Asn^{175} and His^{159} interact directly via hydrogen bonding, one of the obvious roles of Asn^{175} could be to stabilize the thiolate-imidazolium form of papain. It has been suggested that the proximity of the active site cysteine and histidine residues could be one of the most important factors contributing to the formation of an ion pair and that the proton affinities of Cys^{25} and His^{159} at the active site of papain are strongly sensitive to the geometry of these residues (17, 19). Consequently, Asn^{175} could stabilize the ion

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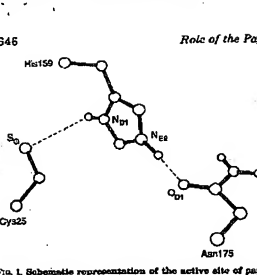

FIG. 1. Schematic representation of the active site of papain showing the catalytic triad residues Cys^{25}, His^{159}, and Asn^{175}. The representation is derived from the crystal structure of Drenth *et al.* (7). In the crystal structure, the active site cysteine is oxidized, and therefore the precise relative orientations of the Cys^{25} and His^{159} side chains in the non-oxidized enzyme might differ from the illustrated orientations.

pair by keeping the imidazole ring of His^{159} in a favorable orientation.

There has been no quantitative experimental study addressing the role of the asparagine residue in the catalytic triad of cysteine proteases. In a preliminary study using random mutagenesis and screening of mutants, we have shown that replacement of Asn^{175} by several amino acids results in a significant loss of activity (20). However, due to the relatively low sensitivity of the assay, this system can unambiguously detect only mutants with activity similar to wild-type papain. In addition, enzyme inactivation could occur for mutant enzymes that have a decreased stability under the relatively drastic conditions used to activate the enzyme precursors (low pH and high temperature). The screening system we used cannot readily distinguish between a decrease in catalytic activity and a decrease in protein stability. In this study, the role of Asn^{175} at the active site of cysteine proteases was investigated by a detailed kinetic and thermal characterization of papain mutants. Mutation of Asn^{175} to a glutamine was chosen due to our previous observation that this mutation generates an enzyme that retains some activity (20), indicating that the conservative substitution of Asn^{175} by Gln is tolerated in the active site of papain. Complete removal of the hydrogen bonding capability of the side chain of residue 175 was accomplished by an Asn^{175} → Ala change.

EXPERIMENTAL PROCEDURES

Expression and Purification of Papain Mutants—Expression of wild-type papain and of the Asn^{175} → Gln and Asn^{175} → Ala mutant proenzymes in *Saccharomyces cerevisiae* has been reported recently (20). Yeast cells from 1 liter of culture (5 × 10^9 cells/ml) were collected by centrifugation and resuspended in 20 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA to yield a final volume of about 3.5 ml. The cells were lysed using a French press (20,000 p.s.i.) and the cellular debris removed by a 10-min, 15,000 × *g* centrifugation. Propapain present in the supernatant was converted to mature papain by limited proteolysis with subtilisin BPN′ (Sigma). The soluble extract was incubated for 2–3 h at 37 °C in the presence of 0.1 mg/ml subtilisin. The extract was then changed to pH 5.0 with sodium acetate buffer (20 mM, pH 4.0) and incubated at 55 °C for 15 min. After a 10-min centrifugation at 15,000 × *g*, precipitated proteins were discarded and the supernatant was made 50% ammonium sulfate and kept at 4 °C overnight. This suspension was centrifuged at 22,000 × *g* for 20 min and the protein pellet resuspended in 4 ml of 100 mM sodium acetate, 1 mM EDTA, pH 5.5. This preparation was used to determine the protein half-life (see below).

The enzymes used for the kinetic characterization were further purified by covalent chromatography using a Chiroprep-Sepharose column (21).

Kinetics of Irreversible Thermal Inactivation—The kinetics of irreversible thermal inactivation of papain variants was determined as described previously (22). Partially purified papain preparations (see above) were adjusted to pH 5.0 with 100 mM phosphate buffer and $HgCl_2$ added to 5 mM. They were incubated at 58 °C for 0–80 min, and the residual papain activity was measured. The $T_{1/2}$ value (the time at which the enzyme has lost half of its activity) was determined from the slope of the linearized form of the data (23).

Aggregate/Soluble Precursor Partitioning and Susceptibility to Protease Degradation—Total yeast extracts (3 ml) were prepared from 75 ml of cultures grown under the conditions defined above. Processing of propapain was prevented with 0.1 mM of E-64 (1-[[(*L*-*trans*-epoxysuccinyl)-*L*-leucylamino]-4-guanidinobutane) (24). The extract was deglycosylated by incubation for 2 h at 37 °C in the presence of 50 mM sodium acetate buffer, pH 5.5, 200 mM β-mercaptoethanol, and 50 milliunits/ml endoglycosidase H (Boehringer Mannheim). An aliquot of the mixture was centrifuged at 15,000 × *g* for 5 min. The supernatant was recovered and the pellet was resuspended in 200 μl of phosphate buffer, pH 6.8. An aliquot of the pellet and supernatant deglycosylated fractions was analyzed by Western blot prior to or following incubation with 0.1 mg/ml subtilisin for 2 h at 37 °C. Quantitative Western blot analyses were performed using two rounds of antigen detection after separation of the proteins in SDS-polyacrylamide gel electrophoresis. Mature and proenzyme forms of papain were detected with an affinity-purified rabbit polyclonal antibody (24). Papain-antibody complexes were labeled with ^{125}I-labeled protein A (Amersham Corp.) and visualized by autoradiography. The antigen was then stained in a second reaction using alkaline phosphatase-conjugated goat anti-rabbit IgGs (Bio-Rad). This procedure facilitates accurate cutting of the immunoreactive bands for radioactivity measurements using an LKB 1282 Compugamma counter.

Kinetic Measurements—The kinetic parameters were obtained as described previously (21). The concentration of active purified enzyme was determined by titration with E-64 (25). Carbobenzoxy-*L*-phenylalanyl-(7-amino-4-methylcoumarinyl)-*L*-arginine (Cbz-Phe-Arg-MCA)[1] was used as a substrate. The reaction conditions consisted of 50 mM phosphate buffer, 0.2 M NaCl, 5 mM EDTA, 10% CH_3CN, pH 6.5. For the determination of pH activity profiles, 80 mM citrate or 50 mM borate were also used as buffers and the substrate concentration was kept well below the K_m value. Kinetic parameters at optimum pH (6.5) were determined by linear regression of the initial rate (v_i) data to plots of v_i/s *versus* v_i (Hanes plots). The pH activity profiles were analyzed according to the model of Reaction 1 by nonlinear regression of the data to the corresponding equation (Equation 1).

$$E\!H_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E \qquad \downarrow (k_{cat}/K_m)^{lim}$$

REACTION 1

$$(k_{cat}/K_m)^{obs} = \frac{(k_{cat}/K_m)^{lim}}{1 + \frac{[H^+]}{K_1^{app}} + \frac{K_2^{app}}{[H^+]}} \qquad \text{(Eq. 1)}$$

In this equation, $(k_{cat}/K_m)^{obs}$ represents the experimentally determined value of the specificity constant and $(k_{cat}/K_m)^{lim}$ is the limiting value determined from nonlinear regression.

Computer Modeling—Computer modeling was used with the Asn^{175} → Gln mutant to predict the orientation of the Gln^{175} side chain. The coordinates of free papain were obtained using the coordinates from the crystal structure of Drenth *et al.* (7). In the model, the oxygen atoms on the oxidized Cys^{25} residue were removed, and AMBER partial charges were assigned randomly such that the active site residues are present in the thiolate-imidazolium ion pair state. In an initial step, the Systematic Search module of Sybyl 6.5 (Tripos Associates, Inc.) was used to carry out a search for sterically allowed conformations of the Asn^{175} → Gln mutant. The Asn^{175} residue was replaced by Gln and the side chain angles χ_1, χ_2, and χ_3 of Gln^{175} were varied by 2-degree increments. Two "groups" of structures (structure 1 and structure 2) were found, both containing an hydrogen bond between the oxygen atom of the Gln^{175} side chain amide and N$^{\epsilon 2}$H of His^{159}. The structures

[1] The abbreviations used are: Cbz-Phe-Arg-βNA, carbobenzoxy-*L*-phenylalanyl-(7-amino-4-methylcoumarinyl)-*L*-arginine; WT, wild-type.

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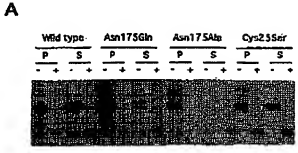

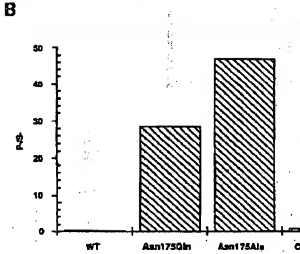

FIG. 2. Segregation of propapain mutants between pellet and supernatant and sensitivity to proteolytic degradation by subtilisin. *Panel A*, autoradiogram of Western blot for the pellet fraction (*P*) and the supernatant fraction (*S*) before (−) and after (+) treatment with subtilisin. The source of the sample is indicated above each series of four samples. The location of mature papain and propapain is indicated in the *right margin* of the autoradiogram. *Panel B*, histogram representing the relative levels of insoluble (*P*−) to soluble (*S*−) proenzymes.

differ by the positioning of the acetamide NH_2 group, which can be either in proximity of Trp^{177} and Trp^{181} (structure 1) or oriented toward the interface between the two domains of papain where it can form a hydrogen bond with the side chain of Ser^{176} (structure 2). Years of these two conformations was energy-minimized in an attempt to predict if one orientation would be favored over the other. Conformational energy terms were calculated using the AMBER force field and partial charges. A distance-dependent dielectric constant, $\epsilon = r$, was used with a residue-based cutoff distance of 8.0 Å. The minimization was carried out to a root mean square gradient of 0.05. Energy calculations were performed on a region delimited by a 12-Å sphere around the C$^{\alpha}$ atom of Gln^{175}, while atoms within a 9-Å sphere around the same C$^{\alpha}$ atom were allowed to move during the minimization. The difference in energy between the two minimized structures was 2.0 kcal/mol in favor of structure 1. However, this value is close to the precision of the calculations and the second conformational state (structure 2) cannot clearly be ruled out. For structure 1, the torsional angles of the Gln^{175} side chain are ϕ = −115.77, ψ = 150.84, χ_1 = −119.87, χ_2 = −56.28, and χ_3 = 77.61, and the Gln^{175} O$^{\epsilon1}$–His^{159} N$^{\epsilon2}$H distance is 2.77 Å. With this conformation, the acetamide N atoms of Gln^{175} are positioned to interact with the π clouds of the two tryptophan residues (Trp^{177} and Trp^{181}). In the alternate structure (structure 2), ϕ = −116.53, ψ = −179.33, χ_1 = −120.28, χ_2 = −57.95, and χ_3 = −92.45, and the Gln^{175} O$^{\epsilon1}$–His^{159} N$^{\epsilon2}$H distance is 2.76 Å.

RESULTS

Pellet / Supernatant Partitioning and Protease Susceptibility of Propapain.—We have investigated the consequences of replacing Asn^{175} upon the solubility of the protein to be detected as a molecule with native properties. During the course of purifying papain, we observed that the yield of mature papain recovered following *in vitro* trans-activation was much lower for mutants at position 175 than for the wild-type enzyme. Using Western blot analysis, we have shown previously that the total amount of propapain produced was not affected by the mutations (22). Therefore, the differences in yield are not consequences of variations in the transcription or translation efficiency or intracellular instability of the proteins. The reduction in yield, which is more pronounced for the Asn^{175} → Ala mutant, could, however, reflect an increased susceptibility to proteolytic degradation by subtilisin in the activation step for the mutants. This suggests that some of the molecules may not be properly folded. Since unfolded proteins are often found to aggregate (26), we have measured the solubility of propapain mutants and the sensitivity of soluble and insoluble fractions to degradation by subtilisin. In the presence of exogenous proteases, the 38-kDa wild-type propapain is fully converted into 24-kDa mature papain (23). This limited proteolytic processing can be easily distinguished from more extensive and less specific degradation of unfolded mutants. A large proportion of propapain mutants at position 175 is found in the pellet fraction (Fig. 2*A*, *lanes P*−), the effect being more pronounced for the Asn^{175} → Ala mutant. The pellet fraction is completely degraded by subtilisin (Fig. 2*A*, *lanes P*+), whereas the soluble fraction (Fig. 2*A*, *lanes S*−) is fully converted to mature papain (Fig. 2*A*, *lanes S*+). The ratio of aggregated to soluble fraction is about 0.3 for the wild-type propapain (Fig. 2*B*). This ratio is close to 30 and about 60 for the Asn^{175} → Gln and Asn^{175} → Ala mutants, respectively. The Cys^{25} → Ser mutant was used as a control and has a behavior similar to that of wild-type propa-

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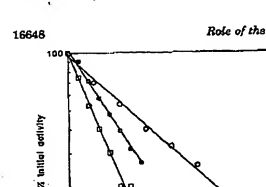

FIG. 3. Kinetics of thermal inactivation of mature papain. Partially purified preparations of WT (○), $Asn^{175} \rightarrow Gln$ (●), and $Asn^{175} \rightarrow Ala$ (□) mature papain were incubated at 82 °C for various periods of time under conditions described under "Experimental Procedures," and the level of remaining activity was measured. Each point is the average value of three (Asn^{175} mutants) or two (wild-type) independent measurements.

pain (Fig. 2, *A* and *B*). Thus, the partitioning of propapain between soluble, protease-resistant fractions and insoluble, protease-susceptible fractions is markedly altered by the replacement of Asn^{175}.

The previous results suggest that mutation at position 175 has a detrimental effect upon the ability of the proenzyme to fold in the cell but that, when the protein is folded, it becomes resistant to proteolytic degradation. However, subtilisin can selectively remove the pro region of the precursor and release mature active papain. To determine if mutations at position 175 affect the stability of mature papain, we have measured the rate of thermal inactivation of papain mutants as defined previously (22). The mature enzyme is known to be highly stable to thermal inactivation, as shown by the half-life of 12.5 ± 0.9 min measured for wild-type papain at 82 °C. For the $Asn^{175} \rightarrow Gln$ and $Asn^{175} \rightarrow Ala$ mutants, the half-life times at 82 °C are 7.3 ± 0.8 min and 4.6 ± 0.3 min, respectively (Fig. 3), indicating that the mutations also have an effect on the thermal stability of the mature enzyme.

Kinetic Characterization—The papain mutants $Asn^{175} \rightarrow Gln$ and $Asn^{175} \rightarrow Ala$ used for kinetic characterization were purified by covalent affinity chromatography. The kinetic parameters at optimum pH (6.5) for hydrolysis of Cbz-Phe-Arg-MCA by the Asn^{175} mutants and wild-type papain are given in Table I. Removal of the Asn^{175} side chain by replacing asparagine by an alanine residue leads to a marked 150-fold decrease in $(k_{cat}/K_M)^{obs}$ at pH 6.5. This effect on activity can be entirely attributed to a decrease in k_{cat}, which is 0.35 s^{-1} for the mutant $Asn^{175} \rightarrow Ala$ as compared to 41.8 s^{-1} for wild-type papain. Mutation of residue 175 to an alanine therefore has a marked effect on the activity of papain. However, if the Asn^{175} residue is replaced by a glutamine, the kinetic parameters for the mutant show relatively little deviation from those of wild-type. The $(k_{cat}/K_M)^{obs}$ value for $Asn^{175} \rightarrow Gln$ is 135 × 10^3 M^{-1} s^{-1}, a value only 3.4-fold lower than that of wild-type enzyme. Replacing an Asn by a Gln can be considered as an insertion of an extra methylene group in the side chain of the Asn residue, and the enzyme seems to be able to tolerate this modification as determined by the kinetic properties of the enzyme.

The influence of pH on $(k_{cat}/K_M)^{obs}$ for the $Asn^{175} \rightarrow Gln$ and $Asn^{175} \rightarrow Ala$ mutants is illustrated in Fig. 4 (*A* and *B*, respectively). The pH activity profiles of the mutants are significantly narrower than that of wild-type papain (represented by a *dashed line* in the figures), particularly in the case of the $Asn^{175} \rightarrow Ala$ mutant. Once again, replacement of Asn^{175} by an alanine has a more pronounced effect than mutation to a glutamine. The pH activity profiles of the mutant enzymes can be fitted to an equation describing a model where two pK_a values (*i.e.* two ionizable groups) are considered, one for each limb of the bell-shaped profile. For the wild-type enzyme, the profile is best described by a three-pK_a model, the additional ionizable group influencing the activity of the enzyme only in the low pH region (21). Due to the precision of our experimental measurements with the mutant enzymes, we cannot rule out the possibility that a third ionizable group also modulates the activity in the acid limb of the pH activity profiles for the mutants. However, this group would have only a small effect on activity, as observed with wild-type enzyme (21). In addition, since the low pH limb of the profile for the mutant enzymes is displaced to higher pH values, the third ionizable group might not modulate the activity in the pH range where the $Asn^{175} \rightarrow Ala$ and $Asn^{175} \rightarrow Gln$ mutants are active. The value of pK_{2a}^{obs}, which is 4.54 for wild-type papain (see Table I) increases to 5.42 for $Asn^{175} \rightarrow Ala$. Similarly, pK_{2a}^{obs} is seen to decrease significantly from 8.45 in wild-type papain to 7.75 in the $Asn^{175} \rightarrow Ala$ variant. These variations in the pK_a values of the ionizable groups that modulate the activity of papain are the largest observed so far with mutants of this enzyme.

Considerations on the Stability of the Thiolate-Imidazolium Form of Papain—It is generally accepted that the active form of papain consists of a thiolate-imidazolium ion pair (9–12). The stability of this ion pair is considered to be very sensitive to its environment. In the present study, a perturbation of the ion pair is a likely possibility since Asn^{175} interacts directly with one of its members. The $O^{\delta 1}$ atom of Asn^{175} is hydrogen-bonded to $N^{\epsilon 2}$ of His^{159}, and this interaction could be important for stabilization of the thiolate-imidazolium ion pair form of the active site residues in cysteine proteases. It has been shown previously that any factor influencing the ion pair stability will consequently have an effect on the observed activity ($(k_{cat}/K_M)^{obs}$) of the enzyme (27). In the same study, it was also shown that using certain assumptions, the effect of a mutation on ion pair stability and on the intrinsic activity (k_{cat}/K_M) of papain can be dissected out by a detailed analysis of the pH activity profile.

The model introduced to establish the relationship between the stability of the thiolate-imidazolium ion pair and the measured kinetic parameters has not been applied so far to the characterization of mutations involving directly one of the active site residues (Cys[25], His^{159}, or Asn^{175}). To be applicable to the analysis of mutations at position 175 of papain, the equations deduced from the model need to be expanded. In its simplest form, the model describing the ionization pathways of the active site residues is represented in Fig. 5. The four prototropic states of the active site residues are considered, and K_I is an equilibrium constant used to describe the conversion of the neutral form (−SH, −Im) to the ion pair form ($-S^-$, $-ImH^+$) of these residues. In the previous study (27), equations were derived assuming that the difference between pK_{1a} and pK_{2a}, the intrinsic pK_a values for the ionization of Cys^{25} and His^{159} in absence of factors stabilizing the ion pair, is the same for both wild-type and mutant enzymes. In the present study, this condition is not necessarily met and a more general equation has to be introduced (Equation 2),

$$\Delta\Delta pK = 2 - \log\left(\frac{K_{I_{mut}} + 1}{K_{I_{WT}} + 1}\right) + \Delta pK_a - \Delta pK_a \qquad \text{(Eq. 2)}$$

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TABLE I
Kinetic parameters for hydrolysis of Cbz-Phe-Arg-MCA by papain variants

Enzyme	k_{cat}	K_M	$(k_{cat}/K_M)^{obs}$	pK_1^{obs}	pK_2^{obs}	ΔpK	$(k_{cat}/K_M)^{obs}$
	s^{-1}	mM	$10^{-3} \times M^{-1} s^{-1}$				$10^{-3} \times M^{-1} s^{-1}$
Wild-type[a]	41.8 ± 8.8	0.089 ± 0.006	484 ± 44	4.54 ± 0.29	8.45 ± 0.02		682 ± 46
Asn^{175} → Ala	0.38 ± 0.15	0.134 ± 0.042	3.08 ± 0.80	5.43 ± 0.21	7.75 ± 0.19	~1.59	2.6 ± 0.9
Asn^{175} → Gln	18.3 ± 4.5	0.140 ± 0.058	135 ± 27	5.02 ± 0.04	7.86 ± 0.18	~1.07	145 ± 29

[a] For wild-type papain, the pH activity profile in the acid limb is best described by two pK_a values of 3.6 and 4.54 (40). The highest of the two pK_a values is considered to represent ionization of the same group as the one modulating the activity for the mutant enzymes and was therefore assigned to pK_1^{obs} in the table (see text).

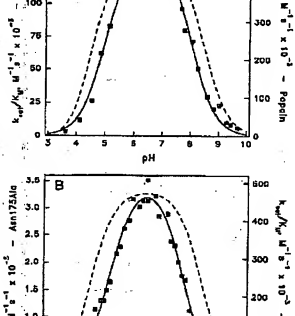

FIG. 4. pH dependence of $(k_{cat}/K_M)^{obs}$ for the Asn^{175} → Gln (*panel A*) and Asn^{175} → Ala (*panel B*) mutants. The *solid line* represents the best fit to Reaction 1, obtained by nonlinear regression of the data to Equation 1. The corresponding pH activity profile for wild-type papain is included for comparison (*dashed line*).

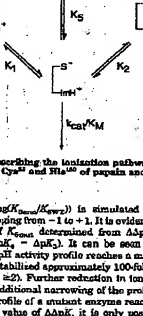

FIG. 5. Model describing the ionization pathways for the active site residues Cys^{25} and His^{159} of papain and cysteine proteases in general.

where $\Delta pK = (pK_2^{obs} - pK_1^{obs})_{mut} - (pK_2^{obs} - pK_1^{obs})_{WT}$, the variation in width of the pH activity profile on going from wild-type papain to the mutant enzyme; $\Delta pK_3 = (pK_{3mut} - pK_{3WT})$ and $\Delta pK_4 = (pK_{4mut} - pK_{4WT})$ are the variations in the intrinsic pK_a values, pK_3 and pK_4, resulting from the mutation; K_{3mut} and K_{3WT} represent the equilibrium constant K_3 for the mutant and wild-type enzymes, respectively. Since the value of $(\Delta pK_4 - \Delta pK_3)$ could be non-negligible, it may partially mask or amplify the effect of a variation in K_3 on the width of a pH activity profile. In Fig. 6A, the variation of ΔpK with ion pair stability (*i.e.* $-\log(K_{3mut}/K_{3WT})$) is simulated for values of $(\Delta pK_4 - \Delta pK_3)$ ranging from −1 to +1. It is evident from Fig. 6A that the value of K_{3mut} determined from ΔpK is strongly dependent on $(\Delta pK_4 - \Delta pK_3)$. It can be seen also that the narrowing of the pH activity profile reaches a maximum when the ion pair is destabilized approximately 100-fold or more (*i.e.* $-\log(K_{3mut}/K_{3WT}) \geq 2$). Further reduction in ion pair stability does not lead to additional narrowing of the profile, and when the pH activity profile of a mutant enzyme reaches this theoretical maximum value of ΔpK, it is only possible to put a higher limit to the value of K_{3mut}. However, the relationship between $(k_{cat}/K_M)^{obs}$ and K_3 is linear when the ion pair is significantly destabilized (Fig. 6B), indicating that perturbation of the ion pair will contribute to a decrease in $(k_{cat}/K_M)^{obs}$ even past the limit where no further effect on pH activity profiles is discernible. Therefore, caution has to be used when interpreting results of pH activity measurements in terms of perturbation of the stability of the ion pair form of the active site residues.

It is important to dissect out the contribution of ion pair stability to the measured kinetic parameters when trying to elucidate the role of Asn^{175} in the catalytic mechanism of papain. From the above considerations, it is obvious that this cannot be accomplished in a straightforward manner to yield a definitive answer. However, the data can be interpreted to define limits to the contribution of Asn^{175} to various aspects of the catalytic mechanism. With the Asn^{175} → Gln mutant, the hydrogen bond between the side chains of residues 175 and 159 is believed to be maintained (see below), and in a first approximation we can consider that $(\Delta pK_4 - \Delta pK_3) = 0$ for this mutant. Since $\Delta\Delta pK = -1.07$ for Asn^{175} → Gln (Table I), we can calculate that $K_{3mut} = 0.08$ and replacement of Asn^{175} by a glutamine causes a 7.6-fold destabilization of the ion pair form

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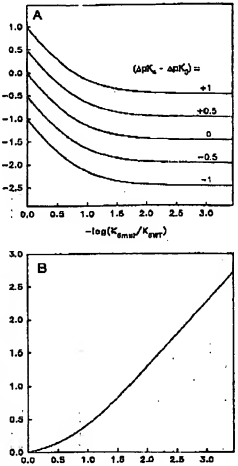

FIG. 6. Simulated curves illustrating the relationships between kinetic parameters and ion pair stability. *Panel A*, relationship between the variation in width of a pH activity profile ($\Delta\Delta pK$) and the perturbation of ion pair stability for various values of ($\Delta pK_a - \Delta pK_a$). *Panel B*, relationship between the variation in the experimentally determined activity (*i.e.* kinetic k_{cat}/K_M value obtained from pH activity profiles) and the perturbation of ion pair stability.

compared to wild-type papain. Knowing the effect of the mutation on the ion pair stability, we can now calculate the effect on the intrinsic activity of the enzyme. To do this, we can use the previously determined equation (Equation 3),

$$(k_{cat}/K_M)^{Gln}_{mut} = (k_{cat}/K_M)_{WT} \cdot f \cdot \left(\frac{K_{amut}}{K_{awt}} + 1\right) \qquad \text{(Eq. 8)}$$

where f represents the effect of a mutation on the intrinsic activity of the enzyme (*i.e.* the ratio of the intrinsic k_{cat}/K_M for mutant over wild-type papain), $(k_{cat}/K_M)_{WT}$ is the intrinsic value of k_{cat}/K_M for wild-type papain, and $(K_{amut}/K_{awt} + 1)$ reflects how the perturbation of K_a resulting from a mutation will affect the measured specificity constant (27). For Asn^{175} → Gln, $f = 0.67$ (Table II), indicating that the intrinsic activity of the mutant is only 1.5-fold lower than that of wild-type papain.

To account for the possibility that ($\Delta pK_a - \Delta pK_a$) might not be negligible for the Asn^{175} → Gln mutant, limits can be put on

TABLE II
Contribution of the Asn^{175} *mutations to ion pair stability and intrinsic activity*

Enzyme	K_a	K_a/K_{awt}	f	$(\Delta pK_a - \Delta pK_a)$
Wild-type	4.4	2200		
Asn^{175} → Gln[a]	0.08	290	0.67	0
Asn^{175} → Gln	4.4	2200	0.30	−1.07
Limiting case 1[b]				
Asn^{175} → Gln	0.33	165	1	+0.16
Limiting case 2[c]				
Asn^{175} → Ala	4.4	2200	0.0072	−1.58
Limiting case 1[b]				
Asn^{175} → Ala	0.0060	3.0	1	−0.12
Limiting case 2[c]				

[a] Calculations done assuming that ($\Delta pK_a - \Delta pK_a$) = 0 for the Asn^{175} → Gln mutant.
[b] Calculations done assuming that the Asn^{175} → Gln or Ala mutation has no effect on ion pair stability, *i.e.* $K_{amut} = 4.4$.
[c] Calculations done assuming that the Asn^{175} → Gln or Ala mutation has no effect on the intrinsic activity, *i.e.* $f = 1$.

the contribution of Asn^{175} to the catalytic mechanism by considering that ion pair destabilization is responsible for none or all of the observed variation in activity. In the first case where the stability of the ion pair is considered not to be affected by the mutation, $K_{amut} = 4.4$ and the decrease in activity is due in totality to a decrease in intrinsic activity of the enzyme. With $\Delta\Delta pK = -1.07$, we can calculate ($\Delta pK_a - \Delta pK_a$) and f using Equations 2 and 3, respectively. In the second limiting case, we consider that all of the effect on the experimentally determined specificity constant originates from a perturbation of the ion pair and that mutation of Asn^{175} to Gln has no influence on the intrinsic activity of the enzyme, *i.e.* $f = 1$. The results using both assumptions, given in Table II, place reasonable limits on the magnitude of the effect that mutation of Asn^{175} to a glutamine can have on the intrinsic activity and on the ion pair stability of papain (assuming of course that the Asn^{175} → Gln mutation does not stabilize the thiolate-imidazolium ion pair or increase the intrinsic activity of the enzyme). It can be seen in Table II that the conclusions are similar to when the variation in ($\Delta pK_a - \Delta pK_a$) was considered negligible, *i.e.* the mutation has only a small effect on ion pair stability and/or intrinsic activity.

For the Asn^{175} → Ala mutant, only the two limiting cases were considered since the probability that the value of ($\Delta pK_a - \Delta pK_a$) is affected by the mutation is much higher. In the first limiting case (no effect on ion pair stability), the value of $f = 0.0072$ indicates that the intrinsic activity of the mutant Asn^{175} → Ala is lower than that of papain by a factor of no more than 140. By considering that the mutation has no effect on intrinsic activity (case 2), $K_{amut} = 0.0060$, a value 735 times lower than that of wild-type papain, which places an upper limit to the ion pair destabilization upon mutation of Asn^{175} to Ala.

DISCUSSION

The role of the asparagine residue in the Cys-His-Asn "catalytic triad" of cysteine proteases has been investigated by replacing Asn^{175} in papain with an alanine or a glutamine residue by site-directed mutagenesis. The kinetic data obtained with the substrate Cbz-Phe-Arg-MCA indicate that Asn^{175} can be replaced by a Gln residue without major changes in the specificity constant (k_{cat}/K_M)$^{Gln}_{mut}$, while mutation to an Ala residue leads to a 150-fold decrease in activity. The side chain of a glutamine retains the possibility of forming a hydrogen bond with the side chain of His^{159} and the higher activity of the Asn^{175} → Gln mutant compared to Asn^{175} → Ala could be explained by the existence of such a hydrogen bond. Computer modeling indeed suggests that the hydrogen bond distances between the side chain amide of residue 175 and His^{159} in

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wild-type papain can be maintained in the Asn^{175} → Gln mutant. When the possibility of residue 175 forming such a hydrogen bond to His^{159} is removed, *i.e.* by mutating Asn^{175} to an alanine, the catalytic efficiency is reduced by about 2 orders of magnitude. However, the Asn^{175} → Ala mutant still hydrolyzes the substrate Cbz-Phe-Arg-MCA at a rate much higher than the non-catalytic rate; therefore, Asn^{175} cannot be considered as an essential catalytic residue in the cysteine protease papain.

A significant fraction of papain exists with the Cys^{25} and His^{159} residues as an ion pair at neutral pH, and from theoretical considerations Asn^{175} has been proposed to stabilize the thiolate-imidazolium ion pair at the active site of papain (19). For a linear peptide containing non-interacting cysteine and histidine residues, if K_{app} is used to designate the ratio of the concentration of the peptide where both side chains are ionized to the concentration where both side chains are neutral, then it can be shown that $\log(K_{app}) = (pK_2 \text{ His} - pK_1 \text{ Cys})$. By using 9.1 and 6.4 for the pK_a values of cysteine and histidine, respectively (28), we obtain a value of 0.0020 for K_{CH}. The value of the corresponding equilibrium constant in papain between the ion pair form and the neutral form of the active site residues has been estimated at 4.4 (27). Therefore, in wild-type papain the ion pair is approximately 2200-fold more stable than if the Cys and His residues were non-interacting in a linear peptide (K_{ip}/K_{CH} in Table II). The mutation of Asn^{175} to Gln is accompanied by an 8-fold decrease in the stability of the thiolate-imidazolium ion pair. Once the ion pair is formed, there is virtually no difference in activity between Asn^{175} → Gln and wild-type papain ($f = 0.87$), suggesting that the advantage of having an Asn at position 175 over a Gln is mainly to stabilize the ion pair. If limiting cases are considered, the value of K_3 can decrease by up to 13-fold while the effect on intrinsic activity is of no more than 3-fold ($f = 0.30$). For the Asn^{175} → Ala mutant, a major perturbation (narrowing) of the pH activity profile is observed and the kinetic data can only be used to put limits to the magnitude of the effects on intrinsic activity and ion pair stability. For example, if the replacement of Asn^{175} by an alanine has a negligible effect on the intrinsic activity of the enzyme (case 2 in Table II), the ion pair stability would be decreased by 735-fold ($K_3 = 0.0060$ compared to 4.4 for wild-type papain). The value of 735 is only 2 times that of K_{CH} for non-interacting residues in a linear peptide. For case 1, where the decrease in observed activity is suggested to be entirely due to a decrease in intrinsic activity, the mutation would have no effect on ion pair stability. It must be noted, however, that in limiting case 1, a relatively high value of $(\Delta pK_2 - \Delta pK_1) = -1.83$ is needed to account for the experimental data. It is most likely that the variations in kinetic parameters observed for the Asn^{175} → Ala mutation are the result of a combination of effects on ion pair stability and intrinsic activity, *i.e.* intermediate between cases 1 and 2.

As discussed above, it is difficult to dissect out the relative effect of the mutation on ion pair stability and intrinsic activity for the Asn^{175} → Ala mutant. However, it is interesting to note that, according to the model linking ion pair stability to pH activity profiles (27), we would expect a strong perturbation in ion pair stability to be accompanied by an important narrowing of the pH activity profile. The quantitative interpretation of pH activity data depends, however, on the correct assignment of pK_a values to active site groups of the enzyme. Recently, the possibility that the increase in k_{cat}/K_M at low pH shown in Fig. 4 could be the result of ionizations other than that of Cys^{25} in the papain molecule was raised (29). If this is the case, the pK_a values measured would not reflect ion pair formation. Changes in the pH activity profile would be the result of variations in reactivity of different protonic forms of the enzyme, without variations in the pK_a values of the groups that modulate activity. This model is relatively complex and requires a large number of parameters to describe the pH activity profiles. Even though this possibility cannot be ruled out unequivocally, we believe that ion pair destabilization leading to narrowing of pH activity profiles is the most likely explanation for our results. The fact that both the acid limb and basic limb pK_a values are affected by the mutations provides a strong argument in favor of a perturbation of ion pair stability. A probable (but not necessary) consequence of ion pair destabilization is that both the acid and basic limbs will be affected. Even though the assignment of the ionization of Cys^{25} to either one of the two pK_a values observed in the acid limb of the pH activity profile for the wild-type enzyme cannot be made unambiguously, as concluded previously (27), the data presented for mutants of Asp^{158} by Ménard *et al.* (27) and for mutants of Asn^{175} (this paper) can all be rationally explained by considering electrostatic effects and ion pair perturbation on a relatively simple model considering in a first approximation only one active form of the enzyme and three ionizable groups. Although more complex explanations cannot be ruled out, we continue to favor the simplest model that fully accounts for the experimental results presented in this report and all related reports originating from this laboratory. Experiments are in progress, however, to clarify this point.

The geometry of the active site Cys-His-Asn residues in cysteine proteases is very similar to that of the corresponding Ser-His-Asp residues forming the catalytic triad of serine proteases (30). Replacement of the Asn^{175} side chain in papain by that of an Ala residue is evaluated to decrease the intrinsic activity of the enzyme by a factor of no more than 140. For serine proteases, mutation of the Asp residue to Ala (for subtilisin) and Ser (for trypsin) resulted in approximately 10^4-fold reductions in enzymatic activity (31, 32). However, an Asn^{102} → Asp mutant of trypsin displayed only 10^2- to 10^3-fold decreases in activity, depending on the nature of the leaving group of the substrate (33). This latter mutation is peculiar in that the side chain can form a hydrogen bond with the active site His residue (34). In addition, it has been shown that even though the presence of the negative charge adjacent to His^{57} in trypsin is important for activity, its precise location is not critical. Indeed, an alternate geometry for the catalytic triad of serine proteases has been proposed (32). The latter two results indicate that certain modifications of the catalytic triad in serine proteases are tolerated.

The magnitude of the apparent contribution to enzymatic activity of the Asn residue in cysteine proteases and the corresponding Asp residue in serine proteases may reflect basic differences in the catalytic mechanism of the two classes of enzymes (35). In the case of serine proteases, the formation of the transition state and tetrahedral intermediate is accompanied by charge separation, and it has been suggested that the negative charge on the aspartate can help this process through electrostatic stabilization, therefore contributing to catalysis (36). In cysteine proteases, the Asn residue in the catalytic triad might be of importance for stabilizing the ion pair form of the catalytic residues (*i.e.* the ground state of the enzyme) by contributing to maintain the active site residues in a favorable geometry. In contrast to serine proteases, charge separation is already present in the ground state and generation of the transition state and tetrahedral intermediate causes only a rearrangement of the charges. In addition, the Asn residue could play a role in catalysis through the orientational effect of the H bond to His^{159}. This hydrogen bond allows rotation of the His^{159} side chain to orient the imidazole group in a proper position to act as a proton donor to the leaving group of the

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substrate. The fact that a C-S bond is weaker than a C-O bond and that the thiolate anion is a very good leaving group can explain the necessity of such a step in cysteine proteases. Therefore, the full catalytic power of the triad might be better exploited in the hydrolysis of non-activated peptide bonds, whereas the activity of the Asn^{175} mutants of papain was measured against a small activated peptidyl substrate. For this reason, the influence of the mutations on activity against protein substrates might be more important than the measured effects with the substrate Cbz-Phe-Arg-MCA, but the very low amount of enzyme available from the expression system precludes such studies. It must be noted also that preliminary results with cathepsin S (data not shown) show that mutation of Asn^{175} has a stronger effect on activity than that observed for papain, indicating that the magnitude of the Asn^{175} contribution to enzymatic activity might differ from one cysteine protease to another.

Highly conserved residues at the active site of enzymes are often regarded as being essential for activity. For the cysteine proteases, it is difficult to account for the strict conservation of Asn^{175} based exclusively upon enzymatic activity, given the relatively modest effect of amino acid substitution at position 175. Indeed, our results show that presence of a Gln at position 175 is almost neutral with respect to the enzyme activity. However, within a large data base of cysteine protease sequences, no residue is found other than an asparagine (37). The strict conservation of Asn^{175} might therefore be the consequence of properties in addition to the catalytic activity of the enzyme. Wild-type propapain accumulates in the yeast cell vacuole (20) mostly as a soluble, protease-resistant species. Replacement of Asn^{175} by either a Gln or Ala increased the fraction of insoluble, protease-susceptible propapain, suggesting that these mutations alter the ability of the protein to fold into a functional protease precursor. In addition, the mature papain mutants resulting from the processing of the properly folded proenzymes have an increased rate of thermal inactivation, indicating that the mutations affect the thermal stability of the mature enzyme. The acetamide H atoms of Asn^{175} in wild-type papain interact with the aromatic rings of Trp^{177} and Trp^{181}, and perturbation of these interactions in the Asn^{175} → Gln mutant could contribute to the decrease in stability of the enzyme. The computer modeling experiments indicate that the Gln^{175}-His^{159} hydrogen bond can be formed with or without perturbation of the interactions with the Trp residues and, therefore, cannot unambiguously support or refute this hypothesis. A similar structural role has been established recently for the catalytic histidine residue at the active site of phospholipase A_2 (38). Our results indicate that in addition to its contribution to the catalytic properties of the enzyme, Asn^{175} participates in the folding pathway (39) and in the thermal stability of the folded protein. The Asn^{175} residue in cysteine proteases could constitute another example of the conservation of an active site residue resulting from a combination of functional and structural constraints.

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EXHIBIT B

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New Peptidic Cysteine Protease Inhibitors Derived from the Electrophilic α-Amino Acid Aziridine-2,3-dicarboxyli Acid

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Received October 23, 1998

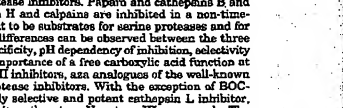
Three different types of peptides containing aziridine-2,3-dicarboxylic acid (Azi) as an electrophilic α-amino acid at different positions within the peptide chain (type I, N-acylated aziridines with Azi as C-terminal amino acid; type II, N-unsubstituted aziridines with Azi as N-terminal amino acid; type III, N-acylated bispeptidyl derivatives of Azi) have been synthesized and tested as inhibitors of the cysteine proteases papain, cathepsins B, L, and H, and calpains I and II, as well as against several serine proteases, one aspartate, and one metalloprotease. All aziridinyl peptides are specific cysteine protease inhibitors. Papain and cathepsins B and L are inhibited irreversibly, whereas cathepsin H and calpains are inhibited in a non-time-dependent manner. Some compounds turned out to be substrates for serine proteases and for the metalloprotease thermolysin. Remarkable differences can be observed between the three different types of inhibitors concerning stereospecificity, pH dependency of inhibition, selectivity between different cysteine proteases, and the importance of a free carboxylic acid function at the aziridine ring for inhibition. Above all type II inhibitors, aza analogues of the well-known epoxysuccinyl peptides, are potent cysteine protease inhibitors. With the exception of BOC-Leu-Gly-(*S,S*+*R,R*)-Azi-(OEt)$_2$ (28a+b), a highly selective and potent cathepsin L inhibitor, N-acylated aziridines of type I are weaker inhibitors than type II or type III compounds. The observed results can be explained by different binding modes of the three types of inhibitors with respect to their orientation in the S- and S′-binding sites of the enzymes. Furthermore, the presence of a protonated aziridine N modifies the binding mode of type II inhibitors.

Introduction

The papain superfamily of cysteine proteases includes a variety of enzymes with closely related amino acid sequences and overall folding structures.[1] Among them are vacuolar plant enzymes (e.g., papain),[2] protozoan enzymes[3] (e.g., cruzipain, falcipain), and mammalian lysosomal cathepsins[4] (e.g., cathepsins B, L, H) and cytoplasmatic calpains.[5] The mammalian cysteine proteases are involved in a variety of pathological processes including dysregulated protein turnover such as muscular dystrophy,[6] bone resorption,[7] growth and malignancy of tumors,[8] and myocardial infarct.[9] Therefore these enzymes are promising targets for the development of inhibitors as therapeutic agents.

A number of irreversible and selective cysteine protease inhibitors have been developed. Most of them exhibit a peptide segment for recognition by the enzyme and an electrophilic building block for reaction with the cysteine residue of the enzyme's active site as common structural features. Examples are diazomethyl ketones,[10] fluoromethyl ketones,[11] acyloxymethyl ketones,[12] *O*-acylhydroxamates,[13] vinyl sulfones,[14] and epoxysuccinic acid derivatives.[15] With regard to the requirements for drugs, the latter are one of the most promising inhibitor classes. Inactivation of cysteine proteases by epoxysuccinyl peptides proceeds from a nucleophilic opening of the epoxide ring leading to alkylated enzymes.[16] Development of these inhibitors was based on the discovery of E-64 (1) (Chart 1) isolated from an *Aspergillus japonicus* culture by Hanada et al.

Chart 1



1 X = O; R^1 = H; R^2 = Agm
E - 64 (HO-(*S,S*)-Epx-Leu-Agm)

2 X = O; R^1 = Et; R^2 = NH - iAm
Loxistatin (Et-(*S,S*)-Epx-Leu-NH-iAm)

3 X = O; R^1 = H; R^2 = NH - iAm
E-64c (HO-(*S,S*)-Epx-Leu-NH-iAm)

5a/b X = NH; R^1 = Et; R^2 = NH - iAm
(a) Et-(*S,S*)-Azi-Leu-NH-iAm
(b) Et-(*R,R*)-Azi-Leu-NH-iAm

6a/b X = NH; R^1 = H; R^2 = NH - iAm
(a) HO-(*S,S*)-Azi-Leu-NH-iAm
(b) HO-(*R,R*)-Azi-Leu-NH-iAm

4 X = O
IBu-NH-(*S,S*)-Epx-Ile(Leu)-ProOH

7a/b X = NH
(a) IBu-NH-(*S,S*)-Azi-Leu-ProOH
(b) IBu-NH-(*R,R*)-Azi-Leu-ProOH

in 1978,[17] cell permeability was improved by replacing the agmatine by uncharged alkyl groups and by esterification of the carboxylate function, e.g., Loxistatin (2) (Chart 1).[18] Even if the esters are 100−1000 times less active than the free acids in vitro, they are used as prodrugs which are easily resorbed and subsequently hydrolyzed to their active forms.[19] These epoxides have been shown to interact in an antisubstrate orientation with the S-subsite of papain and cathepsin B.[20] Cysteine proteases of the papain superfamily prefer substrates

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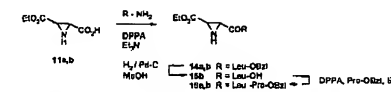
Scheme 1



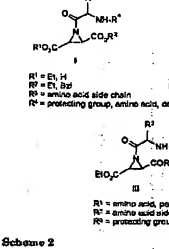
with Phe in the P2-position due to a hydrophobic binding pocket built up by the amino acids Trp67, Phe207, Pro68, Ala160, Val133, and Val157 (papain numbering).[21] Epoxysuccinyl peptides such as 1–3 (Chart 1) bind with their Leu residue into this hydrophobic binding pocket. Epoxides such as 4 interact with the S′-subsite of cathepsin B in a substrate-like mode. In contrast to inhibitors 1–3[22] they are significantly more potent in the *R,R* configuration[23] and exhibit a very good selectivity against cathepsin B.[24] This selectivity results from interaction of the proline carboxylate of 4 with two histidine residues (His110, His111) which are located at the occluding loop of cathepsin B and are responsible for its dipeptidyl peptidase activity.[25] A chimeric epoxysuccinyl peptidic inhibitor has been prepared by Moroder et al.[26] by combining the S′-recognition element of cathepsin B (Leu-Pro) with the inhibiting propeptide sequence of cathepsin B (Leu-Gly-Gly, sequence portion 46–48 of the propeptide) which spans the S-binding pockets in an antisubstrate orientation. This inhibitor is the most potent cathepsin B inhibitor known so far and exhibits a very good selectivity between cathepsin B and cathepsin L.

The three-membered aziridine ring is closely related in structure to the epoxide and is also susceptible to ring opening by nucleophiles.[25] Jones et al.[27] have synthesized and tested the aziridine analogues 5–7 (Chart 1) of the well-known cysteine protease inhibitors EtO-Eps-Leu-NHiAm (2), HO-Eps-Leu-NHiAm (3), and ^{t}BuNH-Eps-ile(Leu)-ProOH (4). Even though these aziridine analogues are also irreversible inhibitors of the cysteine proteases papain, cathepsin B, and cathepsin L, remarkable differences between the epoxides and the aziridines have been found with respect to reactivity, selectivity, stereospecificity, and pH dependency of inhibition.[27] Recently a variety of aziridinyl peptides in which the Leu residue of compounds 5 and 6 has been replaced by Phe have been patented by Takeda Chem. Ind. as powerful cathepsin L inhibitors.[28,29] For these inhibitors naturally occurring epoxide analogues, called cathestatins, are known.[30]

In contrast to the epoxide the aziridine ring can additionally be derivatized at its heteroatom. This offers a greater variability for structure–activity studies. Chimeric inhibitors can not only be built by derivatization of the two carboxylates but also by variations at the aziridine nitrogen. Even though some *N*-alkylated aziridinyl peptides are described in the above-mentioned patents[28,29] and some results concerning *N*-acylated derivatives could already be obtained in the author's laboratory[31] this field has yet to be explored systematically.

In the present paper, both the syntheses and the inhibition profiles of peptides containing aziridine-2,3-dicarboxylic acid as the electrophilic α-amino acid at different positions within the peptide chain (types I–III,

Chart 2



Scheme 2

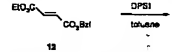


Chart 2) are described. These are classified according to their structural differences: Type I aziridinyl peptides contain the aziridine moiety as the C-terminal amino acid. They are aziridines *N*-acylated with amino acids or peptides. Type II peptides are aza analogues of the known epoxysuccinyl peptides and contain the aziridine-2,3-dicarboxylic acid as the *N*-terminal amino acid. In type III peptides the aziridine is located in the middle of the peptide chain. Although type I and III peptides are both *N*-acylated aziridines, experimental results show differences between these two types which support their classification as two different inhibitor types. The particular peptide moieties used for preparation of the inhibitors have been selected with respect to the substrate specificities[21] of hydrolysis and to known inhibiting sequences,[32] respectively.

Preparation of Inhibitors

The syntheses of the compounds were performed as depicted in Schemes 1–5. Stereoselective synthesis of (*S,S*)- and (*R,R*)-diethyl aziridine-2,3-dicarboxylate (**10a,b**) was carried out according to previously[31,33] described procedures using the reverse configured epoxides (*R,R*)-**8b** and (*S,S*)-**8a**, respectively, as starting materials. The latter were prepared from (*R,R*)- and (*S,S*)-diethyl tartrate, respectively.[23,24]

Half-esters **11a,b** of the aziridine building blocks were prepared by alkaline hydrolysis with 1 equiv of LiOH

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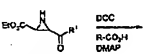
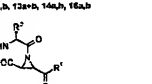
Scheme 3

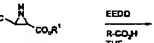


10a,b, 12a+b, 14a,b, 16a,b

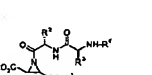


17a,b R¹ = OEt; R² = Bzl; X = BOC
18a R¹ = OEt; R² = Me; X = Z
18e R¹ = OEt; R² = Me; X = BOC
20a R¹ = OEt; R² = iBu; X = Fmoc
21a,b R¹ = Leu-OBzl; R² = Bzl; X = BOC
22a,b R¹ = Leu-Pro-OBzl; R² = Bzl; X = BOC
23a+b R¹ = OBzl; R² = Bzl; X = BOC

Scheme 4

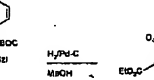


10a,b, 12a+b



24a R¹ = Bt; R² = H; R³ = H; R⁴ = Z
25a,b R¹ = Et; R² = Bzl; R³ = Me; R⁴ = Z
26a' R¹ = Et; R² = Me; R³ = Bzl; R⁴ = BOC
27a+b R¹ = Bzl; R² = Me; R³ = Bzl; R⁴ = BOC
28a+b R¹ = Bzl; R² = H; R³ = iBu; R⁴ = BOC
29a R¹ = Et; R² = Me; R³ = Bzl; R⁴ = Z-Gly

Scheme 5



23a+b 30a+b

monohydrate in ethanol and subsequent protonation by ion exchange.[27] Peptide coupling at the free carboxylate function of the aziridine leading to the type II inhibitors 14a,b and 16a,b could be achieved by the DPPA method, whereby N-protection was not necessary.[27] Both stepwise and fragment condensations were carried out (Scheme 1).

Racemic *trans*-benzylethyl aziridine-2,3-dicarboxylic acid (13a+b) was synthesized by Michael type addition of diphenylsulfimino to the corresponding fumarate 12 (Scheme 2). Most of the aziridinyl peptides derived from aziridines 10a,b were prepared as single diastereomers with *S,S* (a series) as well as *R,R* (b series) configured aziridine ring. Peptides derived from the racemic aziridine 13a+b (Scheme 2) were synthesized as diastereomeric mixtures (a+b series).

Acylation of the aziridine nitrogen with amino acids was carried out via symmetric anhydrides, prepared from 2 equiv of N-protected amino acid and 1 equiv of DCC, under DMAP catalysis (Scheme 3). This yielded the type I compounds 17a,b,[31] 18a,[31] 19a,[31] 20a, and 23a+b and the type III inhibitors 21a,b and 22a,b.

Nitrogen acylation with di- or tripeptides could be performed by fragment condensation using mixed anhydrides of peptide and isobutyl chloroformiate.[30] Since this method leads to racemization within the peptide chain, the one-pot EEDQ[32] procedure was chosen to prepare the type I inhibitors 24a, 25a,b, 26a, 27a+b, 28a+b, and 29a (Scheme 4).

A free carboxylate function at the aziridine ring has been shown to be essential for inhibition by N-unsubstituted derivatives.[27] To examine the role of a free carboxylate group for inhibition by N-acylated derivatives, selective hydrolysis of one ester function of the N-acylated diethyl ester 17a by alkaline or enzymatic methods was investigated. For the alkaline procedure either 1 equiv of LiOH/EtOH or 1 equiv of NaOH/CH$_3$-CN was used. The enzymatic methods were carried out using the following enzymes:[29] PLE, chymotrypsin, trypsin, CCL, PPL, LPR, LRA, and LAN. Alkaline hydrolyses and enzymatic procedures with the serine hydrolases PLE, trypsin, and chymotrypsin, respectively, led to preferential amide hydrolysis. The reasons for the sensitivity of the amide bond to alkaline hydrolysis are disturbed amide resonance[37] and high acidity of the leaving group. Amides containing an aziridine as the amino component cannot form mesomeric structures due to increased ring tension. The pK_a of the aziridinium ion has been determined by Jones et al.[37] with pK_a 3.6 for compound 6a. In the author's laboratory a pK_a of 8.8 ± 0.3[72] was found for compound 11a. PLE is a serine esterase which can also cleave amide bonds and which prefers aromatic residues.[34] Chymotrypsin and trypsin are serine proteases preferring hydrophobic and basic amino acids, respectively, in the P1-position. In both cases, amide and simultaneous ester hydrolysis could be observed. Compound 17a was not hydrolyzed by the above-mentioned lipases. Due to the failure of the selective hydrolysis of one ester function with maintenance of the aziridide structure, another synthetic path had to be chosen. The aziridine building block 13a+b was used as a starting material to allow ester cleavage by nonhydrolytic procedures. The N-acylated aziridinyl peptide 30a+b which contains one free carboxylate function was obtained by N-acylation of building block 13a+b with BOC-Phe via the symmetric anhydride (Scheme 3, compound 23a+b) and subsequent hydrogenolysis of the benzyl ester (Scheme 5).

Results

The second-order rate constants for the inactivation of papain and cathepsins B and L by the aziridinyl peptides of type I, 17−29 and 26−30, which contain the aziridine-2,3-dicarboxylate as the C-terminal amino acid, are shown in Table 1. Since proteases of the papain family prefer Phe in the P2-position, compounds 17a,b, 25a,b, and 26a with a Phe residue at different positions

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Table 1. Second-Order Rate Constants for Inactivation of Papain,[a] Cathepsin B,[b] and Cathepsin L[b] by Type I Aziridinyl Peptides

inhibitor	k_2/K_i (M^{-1} min^{-1})		
	papain	cathepsin B	cathepsin L
BOC-Phe-(*S,S*)-Azi-(OEt)$_2$ (17a)[c]	408 ± 15	125 ± 19	65 ± 12
BOC-Phe-(*R,R*)-Azi-(OEt)$_2$ (17b)[c]	42 ± 9	16 ± 6	16 ± 6
Z-Ala-(*S,S*)-Azi-(OEt)$_2$ (18a)[c]	72 ± 2	67 ± 17	305 ± 26
BOC-Ala-(*S,S*)-Azi-(OEt)$_2$ (19a)	43 ± 5[c]	nd[d]	nd
Fmoc-Leu-(*S,S*)-Azi-(OEt)$_2$ (20a)	65 ± 9[c]	nd	99 ± 10[c]
BOC-Phe-(*S,S*+*R,R*)-Azi-(OEt)(OBzl) (23a+b)[c]	76 ± 9	nd	84 ± 9
BOC-Phe-(*S,S*+*R,R*)-Azi-(OEt)(OH) (30a+b)	505	1230	188 ± 6[c]
	$k_1' = 0.91 \pm 0.013$	$k_1 = 0.83 \pm 0.019$	
	$K_i' = 0.41 \pm 0.07$	$K_i = 0.68 \pm 0.03$	
Z-Gly-Gly-(*S,S*)-Azi-(OEt)$_2$ (24a)	297 ± 56	389 ± 31	317
			$k_1 = 0.087 \pm 0.011$
			$K_i = 0.27 \pm 0.08$
Z-Ala-Phe-(*S,S*)-Azi-(OEt)$_2$ (25a)	177[c] ± 10	126	29 ± 1[c]
		$k_1 = 0.011 \pm 0.001$	
		$K_i = 0.090 \pm 0.001$	
Z-Ala-Phe-(*R,R*)-Azi-(OEt)$_2$ (25b)[c]	21 ± 1	22 ± 1	33 ± 4[c]
BOC-Phe-Ala-(*S,S*)-Azi-(OEt)$_2$ (26a)	1370	455 ± 15[c]	469
	$k_1 = 0.028 \pm 0.0028$		$k_2 = 0.051 \pm 0.019$
	$K_i = 0.020 \pm 0.009$		$K_i = 0.11 \pm 0.08$
BOC-Phe-Ala-(*S,S*+*R,R*)-Azi-(OEt)(OBzl) (27a+b)	1232	443 ± 71[c]	281 ± 2[c]
	$k_1 = 0.020 \pm 0.001$		
	$K_i = 0.016 \pm 0.001$		
BOC-Leu-Gly-(*S,S*+*R,R*)-Azi-(OEt)(OBzl) (28a+b)	349	240 ± 61[c]	2327
	$k_1 = 0.01 \pm 0.001$		$k_2 = 0.028 \pm 0.0015$
	$K_i = 0.069 \pm 0.0012$		$K_i = 0.0088 \pm 0.0015$
Z-Gly-Phe-Ala-(*S,S*)-Azi-(OEt)$_2$ (29a)[c]	523 ± 23	421 ± 100	315 ± 46

[a] pH 6.5. [b] pH 6.0. [c] Measurements were limited to the linear range, with ([I] ≤ K_i due to solubility problems. Therefore, only the second-order rate constant could be obtained. [d] Not determined. [e] k_1 (min^{-1}). [f] K_i (mM).

within the peptide chain were synthesized to evaluate the most favorable distance between the electrophilic building block and P2-recognition element. 20a, 24a, and 28a+b are peptides which contain fragments of the inhibiting procathepsin B sequence Leu-Gly-Gly. These type I inhibitors are irreversible inhibitors of cathepsins B and L and papain. This irreversibility is evident from the time dependency of inhibition in the continuous or dilution assays which were performed to determine the second-order rate constants of inhibition. It was confirmed by dialysis experiments with papain and inhibitors 17a and 11a as well as with cathepsin L and inhibitor 28a+b.

Second-order rate constants for these type I inhibitors are very low and are similar to those found by Albeck et al.[49] for several *erythro*-peptidyl epoxides. Especially cathepsin L is inactivated very weakly by type I aziridines. An interesting and surprising exception in this regard is the inhibition of cathepsin L by mixture 28a+b. Although it is still a weak inhibitor, the second-order rate constant for this diastereomeric mixture is about 10 times higher than all other values found for type I inhibitors. This increase in inhibition results from a 10 times lower binding constant (K_i) and not from a higher first-order rate of inactivation (k_2). A difference between the overall foldings of cathepsins B and L and papain which may be a criterion to explain this outstanding value is the structure of the S2-pocket of cathepsin L.[47] An additional Met161 residue makes its pocket more shallow and narrow compared to those of cathepsin B and papain.

The highest inhibition constants for type I derivatives containing Phe within the peptide moiety are found for compounds 26a and 27a+b with BOC-Phe-Ala as the N-terminal peptide chain.

In contrast to N-unsubstituted aziridines of type II, 14a,b, 5a,b,[27] and 6a,b,[27] for which the second-order rate constants are reported in Table 2, the *S,S* diastereomers 17a and 25a are about 7−10 times more active than their *R,R* isomers 17b and 25b. On the other hand this *stereospecificity* is completely lost in the one observed for the epoxysuccinyl peptides exemplified by compounds 1−3. Another difference between type I aziridines and aziridines without N-derivatization and epoxides 1−3 is the low inhibition improvement by the free carboxylic acid function at the three-membered ring. While 6a,b[27] (Table 2) and even the single aziridine building block 11a (Table 6) exhibit an about 300−600 times higher second-order rate constant for the inhibition of papain than their corresponding esters, values for the inhibition by 30a+b are only 8−10 times higher in comparison to those for 17a,b or 23a+b. Interestingly this inhibition improvement is a result of a higher first-order rate of alkylation (k_2) and not of a lower binding constant (K_i). Taking into consideration that 30a+b is a free acid mixture of diastereomers with *S,S* and *R,R* configured aziridine ring, the inhibition constant for the eutomer may be somewhat higher but nevertheless cannot reach the rates found for N-unsubstituted derivatives.

Prolongation of the peptide chain is normally known to improve inhibition of endoproteinases.[40] However, this is not the case for N-acylated aziridinyl peptides of type I (inhibitor 29a) as well as for the N-unsubstituted ones of type II (inhibitors 16a,b). The highest inhibition constants within the type II inhibitors are observed for *R,R* configured aziridines coupled to a Leu derivative (14b and 5b[27]).

As shown by Jones et al.,[27] N-unsubstituted aziridines are much more potent at low pH values due to protonation of the aziridine nitrogen. The data[27] showed that the neutral form must be at least 100 times less reactive than the protonated form. The true inhibition constants

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Table 2. Second-Order Rate Constants for Inactivation of Papain,[a] Cathepsin B,[b] and Cathepsin L[b] by Type II Aziridinyl Peptides

	k_i/K_i (M^{-1} min^{-1})		
inhibitor	papain	cathepsin B	cathepsin L
EtO-(*S,S*)-Azi-Leu-OBzl (14a)	214 $k_2' = 0.038 \pm 0.0015$ $K_i' = 0.17 \pm 0.03$	41 ± 8[c]	3130 $k_2 = 0.055 \pm 0.009$ $K_i = 0.018 \pm 0.021$
EtO-(*R,R*)-Azi-Leu-OBzl (14b)	1533 ± 41[c]	1607 ± 399[c]	16261 $k_2 = 0.44 \pm 0.0004$ $K_i = 0.027 \pm 0.0001$
EtO-(*R,S*)-Azi-Leu-OH (15b)	63 ± 19[c]	nd	nd[d]
EtO-(*S,S*)-Azi-Leu-Pro-OBzl (16a)	227 $k_2 = 0.021 \pm 0.0002$ $K_i = 0.094 \pm 0.0034$	405 $k_2 = 0.039 \pm 0.02$ $K_i = 0.096 \pm 0.01$	2142 $k_2 = 0.047 \pm 0.006$ $K_i = 0.022 \pm 0.0035$
EtO-(*R,R*)-Azi-Leu-Pro-OBzl (16b)	466 ± 37[c]	188 $k_2 = 0.025 \pm 0.0001$ $K_i = 0.13 \pm 0.0011$	1728 $k_2 = 0.043 \pm 0.0038$ $K_i = 0.025 \pm 0.0037$
EtO-(*S,S*)-Azi-Leu-NHiAm (6a)[e]	180		
EtO-(*R,R*)-Azi-Leu-NHiAm (6b)[e]	3300		
HO-(*S,S*)-Azi-Leu-NHiAm (6a)[e]	$(108 \pm 6) \times 10^3$		
HO-(*R,R*)-Azi-Leu-NHiAm (6b)[e]	$(894 \pm 42) \times 10^3$		
^{t}BuNH-(*S,S*)-Azi-Leu-Pro-OH (7a)[e]	108 ± 6	nd[d]	402 ± 6
^{t}BuNH-(*R,R*)-Azi-Leu-Pro-OH (7b)[e]	nd[d]	780 ± 180	3000 ± 60

[a] pH 6.5. [b] pH 6.0. [c] Measurements were limited to the linear range, with $[I] \leq K_i$, due to solubility problems. Therefore, only the second-order rate constant could be obtained. [d] Not determined. [e] k_i (min^{-1}). [f] K_i (mM). [g] Taken from Jones et al.[27] [h] No time-dependent inhibition.[27]

Table 3. Second-Order Rate Constants for Inactivation of Papain,[a] Cathepsin B,[b] and Cathepsin L[b] by Type III Aziridinyl Peptides

	k_2/K_i (M^{-1} min^{-1})		
inhibitor	papain	cathepsin B	cathepsin L
BOC-Phe-(*S,S*)-(EtO)-Azi-Leu-OBzl (21a)[c]	171 ± 40	122 ± 12	219 ± 16
BOC-Phe-(*R,S*)-(EtO)-Azi-Leu-OBzl (21b)	225 ± 70[c]	267 ± 29[c]	814 $k_2' = 0.0099 \pm 0.0023$ $K_i' = 0.031 \pm 0.0023$
BOC-Phe-(*S,S*)-(EtO)-Azi-Leu-Pro-OBzl (22a)	331 $k_2 = 0.016 \pm 0.0019$ $K_i = 0.05 \pm 0.029$	460 $k_2 = 0.037 \pm 0.0022$ $K_i = 0.082 \pm 0.013$	1210 ± 260[c]
BOC-Phe-(*R,R*)-(EtO)-Azi-Leu-Pro-OBzl (22b)	765 ± 125[c]	1536 ± 440[c]	5896 ± 409[c]

[a] pH 6.5. [b] pH 6.0. [c] Measurements were limited to the linear range, with $[I] \leq K_i$, due to solubility problems. Therefore, only the second-order rate constant could be obtained. [d] k_i (min^{-1}). [e] K_i (mM).

Table 4. Inhibition Constants for the Non-Time-Dependent Inhibition of Cathepsin H,[a] Calpain I,[b] and Calpain II[b]

	K_i (μM)		
inhibitor	cathepsin H	calpain I	calpain II
BOC-Phe-(*R,S*)-Azi-(OEt)$_2$ (17a)	210 ± 19	n[c]	nd[d]
Z-Ala-(*S,S*)-Azi-(OEt)$_2$ (18a)	ni	160 ± 21	nd
(EtO)-(*S,S*)-Azi-Leu-OBzl (14a)	230 ± 35	nd	nd
(EtO)-(*R,R*)-Azi-Leu-OBzl (14b)	ni	ni	nd
BOC-Phe-(*S,S*)-(EtO)-Azi-Leu-OBzl (21a)	93 ± 8	nd	nd
BOC-Phe-(*R,R*)-(EtO)-Azi-Leu-OBzl (21b)	137 ± 11	19 ± 7	42 ± 14

[a] pH 6.5. [b] pH 7.5. [c] No inhibition. [d] Not determined.

Table 5. Second-Order Rate Constants for Inactivation of Papain at Different pH Values[a]

	k/K_i (M^{-1} min^{-1})		
inhibitor	pH 4	pH 6.5	pH 8
16a[b]	61 ± 5	11 ± 0.5	nd[c]
11a[b]	24774 ± 7000	4622 ± 1090	780 ± 85
14a	765 ± 89[d]	169[d]	84 ± 8[d]
17a[b]	72 ± 10	424 ± 32	410 ± 21

[a] Substrate, 1.4–1.5 mM L-BAPA; [E] = 0.5 mg mL^{-1}; [I]: 16a, 0.58–4.7 mM; 11a, 6.5 μM–0.3 mM; 14a, 0.27–1.36 mM; 17a, 0.092–0.37 mM. [b] Measurements were carried out at the linear range, where $[I] \leq K_i$. Therefore, only the second-order rate constant was obtained. [c] Not determined. [d] $k_i = 0.033 \pm 0.002$ (min^{-1}), $K_i = 0.20 \pm 0.038$ (mM).

for the completely protonated inhibitor would be much higher at pH values below pH 4 which cannot be tested because of loss of enzyme activity.[27] The maximum activity pH of 4 determined by Jones et al.[27] for N-unsubstituted derivatives can be confirmed as shown in Table 5. In contrast to N-unsubstituted aziridines the N-acylated aziridine 17a shows nearly identical inhibition constants at pH 6.5 and pH 8. Rich et al.[41] investigated the pH dependency of inhibition of papain by a nonionizable amide of 3. This inhibition was found to depend on two acidic dissociation constants (pK_a's 3.93 and 4.09) of papain. This is in contrast to the inhibition of papain by E-64 (1) for which an acidic dependency and an alkaline dependency were found.[37] The latter result supports the assumption that His159 may play an essential role for inhibition. Since 17a is sensitive to alkaline media, determination of second-order rate constants at pH values above pH 8 is useless. Table 6 reports the pH dependency of inhibition of papain by 17a. Theso values, obtained within a pH range from 5.2 to 8.0, indicate, however, that inhibition

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Table 6. pH Dependency of Inhibition of Papain by 17a

pH	activity (mU mL^{-1}) without 17a[a]	activity (mU mL^{-1}) with 17a[a,b]	% inhibition
5.2	6.54 ± 0.2	1.86 ± 0.03	71.6
6.0	7.33 ± 0.14	1.38 ± 0.1	81.1
6.5	7.38 ± 0.21	0.88 ± 0.09	88.7
7.0	7.33 ± 0.15	0.75 ± 0.15	89.8
8.0	7.31 ± 0.15	0.75 ± 0.2	89.7

[a] Mean values from five assays. [b] [I] = 17 μM; incubation time 80 min; [S] = 1.49 mM L-BAPA; [E] = 0.5 mg mL^{-1}.

by this N-acylated aziridine corresponds to the one found for the amide of 3 rather then to that found for E-64 (1).

Activation of aziridines toward nucleophilic ring opening is possible not only by protonation of the aziridine nitrogen but also by N-acylation.[42] In Table 3 inhibition constants are reported for inhibitors of type III (31a,b, 22a,b). These are bispeptidyl derivatives which bear the aziridine moiety in the middle of the peptide chain and which can be regarded as chimeric inhibitors combining the P1′ and P2′ (Leu-Pro) specificity of cathepsin B with its P2 specificity (Phe). The N-acylation of tripeptide 16b with BOC-Phe to tetrapeptide 22b leads only to a weak inhibition improvement in the case of cathepsin L and papain, but to a 10-fold improvement of inhibition in the case of cathepsin B. No or only a slight increase of inhibition is obtained by N-acylation of the type II inhibitors 14a,b and 16a. Again, in accordance with the results found for type II inhibitors, the *R,R* diastereomers are the more potent, but with a very low eudismic ratio.

When the selectivity between cathepsins B and L and papain was compared, the inhibitors of types II and III which bear peptide chains at one of the carboxylate functions exhibit higher activities against cathepsin L than against cathepsin B or papain. The reverse holds for the type I inhibitors which are, with the one exception of mixture 28a+b already mentioned, only weak cathepsin L inhibitors. Cathepsin H and cathepsin C are not inhibited time dependently by all types of inhibitors. If a reduction in enzyme activity can be observed, K_i values in the upper micromolar range are also observed (Table 4). This non-time-dependent inhibition may be due to extremely low alkylation rates which are also known for the inhibition of these enzymes by epoxysuccinyl peptides.[23,43] For a better inhibition of cathepsin H, inhibitors without an N-terminal protecting group may be required.

To evaluate the selectivity between proteases with different mechanisms of hydrolysis (cysteine → serine → aspartate → metallo) the aziridines 10a, 14b, 17a, 18a, and 21b were tested against the serine proteases chymotrypsin, trypsin, and elastase, the aspartate protease pepsin, and the metalloprotease thermolysin, respectively. As shown by the above-mentioned hydrolysis assays of 17a with several serine hydrolases, this compound does not react as an inhibitor but as a substrate for these enzymes. BOC-Phe and compounds 10a and 11a could be isolated and identified by TLC and IR and NMR spectroscopy. This is in agreement with earlier reports on ester hydrolysis of aziridinecarboxylates by these enzymes.[44,45] In contrast to 21b compound 14b is a substrate for thermolysin which prefers Leu in the P1′-position.[46] In this case 11b and Leu-OBzl could be identified as hydrolysis products. Neither 10a nor 21b could inhibit pepsin.

Summary and Discussion

Even though several similarities, including the exclusive inhibition of cysteine proteases and the irreversibility of inhibition of papain and cathepsins B and L, exist between aziridines and epoxides, their respective behavior as cysteine protease inhibitors shows remarkable differences. These differences may first of all be due to the possible protonation of the aziridine nitrogen in the case of the type II aziridines, thus leading to a different binding mode with an additional water molecule not being necessary and with the positioning of the inhibitor being disturbed by the positively charged group. Second, aziridines of type II are analogues of the epoxides concerning their chemical reactivity, but they are not bioisosters. The epoxide oxygen is a H-bond acceptor, whereas the aziridine nitrogen in most cases reacts as a H-bond donor. Thus totally different interactions with the enzyme could be possible. On the other hand, there exist noteworthy differences between the different types of aziridinyl peptides. These differences concern pH dependency, stereospecificity, and selectivity of inhibition. In contrast to the type II inhibitors H-bonds cannot be built under participation of the aziridine N by the N-acylated type I and III inhibitors. Type I inhibitors which are more active with the *S,S* configured aziridine ring and which should resemble the epoxysuccinyl peptides 1–3 could bind to the S-binding site in a substrate-like mode. Comparison of inhibition constants obtained for the Phe-containing series of inhibitors (compounds 17a,b and 25a,b with 26a and 27a+b) leads to the assumption that the Phe residue of the latter is probably located in the hydrophobic S2-pocket as known from the peptidyl chloromethyl ketone BPACK.[48] The aziridide structure may possibly lead to a more unfavorable inhibitor conformation which could impede the attack of the active site cysteine residue at the aziridine ring carbons and therefore lead to low alkylation rate constants. A superimposition of minimized conformations of the epoxide 2, the type II aziridine 14b, and the type I inhibitor 26a supports this supposition (Figure 1A).[26] However, first results of docking experiments[27] performed with papain and 26a indicate that the flexibility of the molecule nevertheless allows a conformation in which the Phe residue of this inhibitor can bind into the hydrophobic S2-pocket and the N-terminal BOC group can be located within the S′-subsite (Figure 1B).

A comparison of type II compounds 14a,b and 16a,b with the N-acylated type III compounds 21a,b and 22a,b shows that the activation of the aziridine ring to nucleophilic ring opening by N-acylation does in general not lead to an inhibition improvement. Therefore, the main reason for the remarkable increase in inhibitor by protonation in the case of type II inhibitors should be due to improved binding rather than to improved nucleophilic ring opening. Type III inhibitors are new chimeric inhibitors, and similar to epoxides with two peptide chains,[23] a tendency for similar second-order rate constants for the *R,R* and *S,S* configured isomers can be observed. Thus, one can assume that these inhibitors, like the chimeric epoxides, bind to both the S- and S′-binding sites. A new aspect which should be taken into consideration is the possibility that the aziridine ring can be opened by nucleophiles in two different ways. Not only is cleavage of the C–N bond possible but also the cleavage of the C–C bond.[50] C–N cleavage would lead to aspartic acid derivatives, while C–C cleavage would form glycine derivatives. These two

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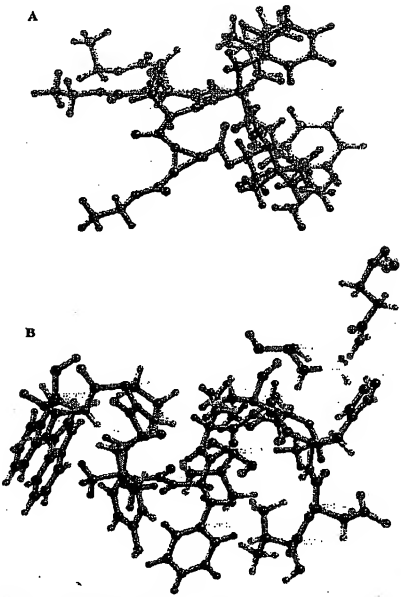

Figure 1. (A) Superimposition of minimized conformations of epoxide 2, type II aziridine 14b, and type I aziridine 26a.[36] (B) Possible binding mode of 26a to the active site of papain.[77]

possibilities may also play a role in the observed differences between epoxides and aziridines on the one hand and between different types of aziridinyl peptides on the other hand.

The exclusive inhibition of cysteine proteases could be explained by the enhanced nucleophilicity of the active site compared to serine proteases as a result of the thiolate–imidazolium ion pair[74] even though the nucleophilicity of serine and cysteine proteases cannot be compared that easily. The "hard" nucleophile OH of the serine proteases attacks the "hard" electrophilic C=O center, while the "soft" S- and ophile attacks the "soft" ring carbon.[75] Another reason for this selectivity may be the opposite active site geometry of serine proteases[21] which could convert aziridinyl peptides from inhibitors into substrates for serine proteases. Starting points to improve the selectivity between different cysteine proteases are the good activities of type II inhibitors and the type I mixture 28a+b against cathepsin L and the remarkable inhibition improvement by the N-acylation of 16b with BOC-Phe to 22b in the case of cathepsin B. A better understanding of the observed differences between epoxysuccinyl peptides and aziridinyl peptides on the one hand and between the three types of aziridines on the other hand will require more

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detailed molecular modeling studies as well as X-ray analysis of enzyme–inhibitor complexes. These studies are in progress. Nevertheless, the present study shows that N-unsubstituted aziridinyl peptides and in special cases N-acylated ones, too, can be highly selective and potent inactivators of cysteine proteases.

Experimental Section

General Methods. Enzymes were purchased from the following companies: papain from *Carica papaya* from Fluka (BioChemika USP, 76220), cathepsin B from bovine spleen from Sigma (C 6286), cathepsin H from human liver (219404), calpain I from porcine erythrocytes (208712) and calpain II from porcine kidney (208715) from Calbiochem, chymotrypsin from bovine pancreas (102307), trypsin from bovine pancreas (124579) and pepsin (7185) from Merck, elastase from porcine pancreas (20929) from Serva. Cathepsin L from *Paramecium tetraurelia*[56] was a gift from Prof. J. Schultz, Department of Pharmaceutical Chemistry, University of Tuebingen, Germany. Pig liver esterase PLE (46063), lipase from *Rhizopus arrhizus* LRA (62305), lipase from *Aspergillus niger* LAN (62294), lipase from *Penicillium roquefortí* LPR (62308), and lipase from hog pancreas PPL (62300) were from Fluka. *Candida cylindracea* lipase type VII CCL (L1754) was from Sigma. All enzymes were used without further purification. All substrates, protected amino acids, and di- and tripeptides were purchased from Bachem. Leu-Pro-OBzl trifluoroacetate and BOC-Phe-AlaOH were prepared by well-established literature procedures.[24,62] Buffer substances were biochemical grade and were purchased from Merck. *N*-Ethylmaleimide was from Aldrich; E-64 was from Boehringer-Mannheim. Reagent grade chemicals were purchased from the following companies and were used without further purification: EEDQ from Novabiochem, DPSI, NaN_3, $LiOH{\cdot}H_2O$, and NH_4Cl from Merck, Ph_3P, DCC, DMAP, and Et_3N from Fluka, DPPA from Aldrich, Pd–C 10% type E 10 N/D from Degussa. All solvents were anhydrous grade and were purchased from Fluka. Brine refers to a saturated aqueous solution of NaCl. Analytical TLC was performed on Merck aluminum sheets (silica gel 60 F_{254}). Compounds that were not visualized by UV light were detected by spraying with Ehrlich's reagent (1 g of *p*-(dimethylamino)-benzaldehyde, 25 mL of HCl concentrated, 75 mL of MeOH) followed by heating. Preparative flash column chromatography was performed using silica gel 60, 40–63 μm, from Merck. Preparative hydrostatic column chromatography was performed using silica gel 60, 63–200 μm, from Merck. Melting points are uncorrected and were obtained on a Mel-Temp II capillary melting point apparatus (Laboratory Devices). Optical rotations were measured on a Perkin-Elmer 241 polarimeter in a thermostated cell. IR spectra were determined in KBr pellets or with NaCl solution cells on a Perkin-Elmer 841 IR spectrophotometer. Mass spectra were measured on a Finnigan MAT 312 (low resolution; EI, 70 eV, 0.8 mA; CI, isobutane or NH_3, 200 eV, 0.5 mA) by Chemisches Laboratorium of the University of Freiburg or on a Voyager-RP biospectrometry workstation by PerSeptive Biosystems, Wiesbaden, Germany (high resolution) (mode, reflector; accelerating voltage, 12 or 15 kV; scans averaged, 15–34; negative ions, off). Elemental analyses were determined on a Perkin-Elmer elemental analyzer 240 by Chemisches Laboratorium of the University of Freiburg. NMR (^1H, ^{13}C) spectra were recorded on a Varian Unity 300 spectrometer (300 and 75.43 MHz, respectively). ^1H NMR chemical shifts are reported in ppm relative to the $CHCl_3$ peak at $\delta = 7.26$ with $CDCl_3$ as solvent and to the DMSO peak ($\delta = 2.49$) with DMSO-d_6 as solvent. ^{13}C NMR chemical shifts are reported in ppm relative to the $CHCl_3$ peak ($\delta = 77.00$) with $CDCl_3$ as solvent. All ^1H NMR assignments were supported by homonuclear decoupling experiments or by 2-D COSY experiments. All ^{13}C NMR assignments were supported by 2-D HETCOR experiments. Coupling constants (*J*) are reported in hertz (Hz).

General Procedures. N-Acylation via Symmetric Anhydrides. N-Protected amino acid (15.5 mmol) was dissolved in 40 mL of dichloromethane at 0 °C; 1.65 g (7.7 mmol) of DCC was added, and the mixture was stirred at 0 °C for 45 min. Insoluble dicyclohexylurea was filtered off, and 7 mmol of aziridine was added in 20 mL of dichloromethane at 0 °C together with a few crystals of DMAP (10 mol %). The reaction was stirred at 0 °C for 1 h and for 3–5 h at room temperature. Dicyclohexylurea was filtered off, and the solvent was removed. The residue was stirred in 10 mL of ethyl acetate for 20 min and again filtered off. The organic layer was washed with saturated $NaHCO_3$ solution (20 mL) and brine (20 mL), dried with $MgSO_4$, and concentrated. The residue was purified for each individual compound by either flash or hydrostatic chromatography as described below.

N-Acylation via Mixed Anhydrides. The coupling reagent EEDQ (426 mg, 1.72 mmol) was added to a solution of N-protected peptide (1.61 mmol) and aziridine (1.61 mmol) in 15 mL of DMF. The mixture was stirred at room temperature for 48 h. The solvent was removed in vacuo; the residue was dissolved in 100 mL of ethyl acetate and extracted with 50 mL of a 5% solution of $NaHCO_3$ and 50 mL of water. The organic layer was dried with $MgSO_4$ and evaporated. The residue was purified for each individual compound by either flash or hydrostatic chromatography as described below.

DPPA-Mediated Peptide Coupling. A stirred solution of carboxylic acid (5 mmol) and C-protected amino acid or peptide (HCl, TFA, or tosylate) (5.2 mmol) in DMF (30 mL) was cooled in an ice–water bath and treated with DPPA (1.2 mL, 5.5 mmol). A solution of Et_3N in 10 mL of DMF (10.5 mmol, 1.43 mL) was added dropwise over a period of 10 min. Stirring and cooling were continued for 10 h. The mixture was diluted with ethyl acetate (150 mL) and washed with 25-mL portions of water (3×), 5% $NaHCO_3$ (1×), and brine (2×). The organic layer was dried with $MgSO_4$ and evaporated in vacuo. The residue was purified for each individual compound by either flash or hydrostatic chromatography as described below.

Catalytic Hydrogenolysis of Benzyl Esters. An evacuated solution of 0.6 mmol of benzyl ester in 30 mL of MeOH and 40 mg of Pd–C (10%) was vigorously stirred at room temperature and atmospheric pressure for 1.5 h (TLC control) under a slow stream of hydrogen. The catalyst was removed by filtration over Celite and washed with methanol (50 mL). The filtrate was evaporated in vacuo, and the residue was recrystallized.

The following compounds were prepared according to literature procedures: **diethyl (2*S*,3*S*)-2,3-epoxysuccinate (8a)**,[54] **diethyl (2*R*,3*R*)-2,3-epoxysuccinate (8b)**,[34] **diethyl (2*S*,3*S*)-aziridine-2,3-dicarboxylate (10a)**,[26] **diethyl (2*R*,3*R*)-aziridine-2,3-dicarboxylate (10b)**,[31] **(2*S*,3*S*)-3-(ethoxycarbonyl)aziridine-2-carboxylic acid (11a)**,[27] **(2*R*,3*R*)-3-(ethoxycarbonyl)aziridine-2-carboxylic acid (11b)**,[27] **benzyl ethyl fumarate (12)**,[69] **diethyl (2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-phenylalanyl]aziridine-2,3-dicarboxylate (17a) (BOC-Phe-(*S*,*S*)-Azi-(OEt)$_2$)**,[31] **diethyl (2*R*,3*R*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-phenylalanyl]aziridine-2,3-dicarboxylate (17b) (BOC-Phe-(*R*,*R*)-Azi-(OEt)$_2$)**,[31] **diethyl (2*S*,3*S*)-1-[*N*-(benzyloxycarbonyl)-(*S*)-alanyl]aziridine-2,3-dicarboxylate (18a) (Z-Ala-(*S*,*S*)-Azi-(OEt)$_2$)**,[31] and **diethyl (2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-alanyl]aziridine-2,3-dicarboxylate (19a) (BOC-Ala-(*S*,*S*)-Azi-(OEt)$_2$)**.[31]

(2*S*,3*S*)- and (2*R*,3*R*)-2-Benzyl 3-ethylaziridine-2,3-dicarboxylate (13a+b) (EtO-Azi-OBzl). DPSI[60] (5.2 g, 0.024 mol) and 5.9 g of benzyl ethyl fumarate (12) (0.02 mol) were dissolved in 60 mL of toluene and heated at 80 °C for 24 h. The mixture was evaporated in vacuo. Column chromatography (silica gel 60, cyclohexane/ethyl acetate, 4/1, $R_f = 0.2$) yielded 1.2 g (25%) of 13a+b as a yellowish solid:[70] mp 52 °C. IR (CCl_4): 3282 (br), 1733, 1497 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.30 (t, $J = 7.0$ Hz, 3 H), 1.85 (bs, 1 H, NH), 2.90 (d, $J = 2.3$ Hz, 1 H, CH-Azi), 2.92 (d, $J = 2.3$ Hz, 1 H, CH-Azi), 4.22 (q, $J = 7.0$ Hz, 2 H), 5.15 (d, $J = 12.0$ Hz, 1 H), 5.25 (d, $J = 12.0$ Hz, 1 H), 7.3–7.45 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 13.81, 35.45, 35.60, 61.71, 67.36, 126.60, 128.22, 128.40, 134.73, 169.22

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Oct-08-2002 03:19pm From-REED SMITH LLP 27N 215-851-1429 T-092 P.025/037 F-094

(double peak). MS (CI, C_4H_{10}): m/z (%) = 306 (1.1) [M$^+$ + C_4H_9], 250 (100) [M$^+$ + 1]. Anal. ($C_{12}H_{19}NO_4$) C,H,N.

***N*-{[(2*S*,3*S*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine Benzyl Ester (14a) (EtO-(*S*,*S*)-Azi-Leu-OBzl).** **11a** was coupled with L-leucine benzyl ester tosylate using the general DPPA procedure. The product was purified by column chromatography on silica gel (ethyl acetate/cyclohexane, 1/1, R_f = 0.44) to give **14a** (760 mg, 42%) as a yellowish viscous liquid: $[\alpha]_D^{27.3}$ = +47.2° (c 1.4, EtOH). IR (CCl$_4$): 3283 (br), 1739, 1675, 1526 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.9 (d, J = 6.1 Hz, 6 H), 1.3 (t, J = 7.08 Hz, 3 H), 1.46–1.7 (m, 3 H), 1.8 (bt, J = 8.5 Hz, 1 H, NH), 2.55 (dd, J = 2.2, 7.8 Hz, 1 H), 2.87 (dd, J = 2.2, 9.0 Hz, 1 H), 4.22 (q, J = 7.08 Hz, 2 H), 4.56–4.65 (m, 1 H), 5.15 (s, 2 H), 6.78 (bd, J = 8.8 Hz, 1 H), 7.28–7.4 (m, 5 H). ^{13}C NMR (CDCl$_3$): δ 14.07, 21.73, 22.79, 24.84, 35.44, 37.40, 41.03, 50.36, 62.20, 67.09, 128.20, 128.41, 128.61, 135.29, 167.75, 170.30, 172.22. MS (EI, 70 eV): m/z (%) = 363 (14) [M$^+$ + 1], 227 (100). Anal. ($C_{19}H_{26}N_2O_5$) C,H,N.

***N*-{[(2*R*,3*R*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine Benzyl Ester (14b) (EtO-(*R*,*R*)-Azi-Leu-OBzl).** **11b** was coupled with L-leucine benzyl ester tosylate using the general DPPA method. The product was purified by column chromatography on silica gel (ethyl acetate/cyclohexane, R_f = 0.39) to give **14b** (638 mg, 35%) as a yellowish viscous liquid: $[\alpha]_D^{23.2}$ = −110.7° (c 1.4, EtOH). IR (CCl$_4$): 3275 (br), 1742, 1670, 1537 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.9 (d, J = 6.1 Hz, 6 H), 1.25 (t, J = 7.0 Hz, 3 H), 1.45–1.8 (m, 4 H, NH, β-H, γ-H), 2.62 (d, J = 2.0 Hz, 1 H), 2.85 (d, J = 2.0 Hz, 1 H), 4.2 (q, J = 7.0 Hz, 2 H), 4.55–4.65 (m, 1 H), 5.13 (d, J = 12.0 Hz, 1 H), 5.18 (d, J = 12.0 Hz, 1 H), 6.65 (bd, J = 8.5 Hz, 1 H), 7.3–7.4 (m, 5 H). ^{13}C NMR (CDCl$_3$): δ 13.83, 21.49, 22.56, 24.58, 35.17, 37.12, 40.72, 50.18, 61.86, 66.80, 127.93, 128.14, 128.35, 135.10, 167.65, 170.02, 171.97. MS (EI, 70 eV): m/z (%) = 363 (9) [M$^+$ + 1], 227 (100). MS (CI, C_4H_{10}): m/z (%) = 363 (100) [M$^+$ + 1]. Anal. ($C_{19}H_{26}N_2O_5$) C,H,N.

***N*-{[(2*R*,3*R*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine (15b) (EtO-(*R*,*R*)-Azi-Leu-OH).** **14b** was hydrogenolyzed using the general hydrogenolysis procedure. Recrystallization from MeOH yielded 95% (155 mg) **15b**: mp 66 °C; $[\alpha]_D^{23}$ = −63.6° (c 0.55, EtOH). IR (KBr): 3374 (br), 1737, 1670, 1544 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.94 (d, J = 5.9 Hz, 3 H), 0.96 (d, J = 6.11 Hz, 3 H), 1.30 (t, J = 7.08 Hz, 3 H), 1.5–1.8 (m, 3 H), 2.71 (d, J = 2.2 Hz, 1 H), 2.89 (d, J = 2.2 Hz, 1 H), 4.23 (q, J = 7.3 Hz, 2 H), 4.5–4.6 (m, 1 H), 5.0 (bs, 2 H, NH, COOH), 6.76 (bd, J = 8.5 Hz, 1 H, NH). MS (CI, C_4H_{10}): m/z (%) = 273 (100) [M$^+$ + 1]. MS (EI, 70 eV): m/z (%) = 273 (12) [M$^+$ + 1], 227 (100). Anal. ($C_{12}H_{20}N_2O_5$·$^1/_2$MeOH) C,N; H: calcd, 7.69; found, 7.23.

***N*-{[(2*S*,3*S*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucyl-(*S*)-proline Benzyl Ester (16a) (EtO-(*S*,*S*)-Azi-Leu-Pro-OBzl).** DPPA-mediated coupling of **11a** with Leu-Pro-OBzl TFA gave, after purification by flash chromatography (cyclohexane/ethyl acetate, 1/2, R_f = 0.19), 51% (1.17 g) of **16a** as a colorless viscous liquid: $[\alpha]_D^{23}$ = −18.3° (c 1.45, EtOH). IR (ethyl acetate): 3271, 1740, 1631, 1551, 1453 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.86 (d, J = 6.35 Hz, 3 H), 0.91 (d, J = 6.35 Hz, 3 H), 1.27 (t, J = 7.1 Hz, 3 H), 1.45 (m, 2 H, β-H Leu), 1.5–1.64 (m, 1 H, γ-H Leu), 1.8–2.1 (m, 4 H, β-H Pro, 2 γ-H Pro, NH Azi), 2.1–2.28 (m, 1 H, β-H Pro), 2.58 (d, J = 1.95 Hz, 1 H), 2.78 (d, J = 2.2 Hz, 1 H), 3.5–3.62 (m, 1 H, δ-H Pro), 3.7–3.8 (m, 1 H, δ-H Pro), 4.2 (q, J = 7.3 Hz, 2 H), 4.54 (m, 1 H, α-H Pro), 4.74 (m, 1 H, α-H Leu), 5.06 (d, J = 12.2 Hz, 1 H), 5.16 (d, J = 12.2 Hz, 1 H), 7.06 (bd, J = 8.0 Hz, 1 H, NH Leu), 7.35 (m, 5 H). ^{13}C NMR (CDCl$_3$): δ 13.97, 21.63 (Leu), 23.19 (Leu), 24.52 (Leu), 24.76 (Pro), 28.84 (Pro), 35.59 (Azi), 37.35 (Azi), 41.47 (Leu), 46.74 (Pro), 48.65 (Leu), 58.82 (Pro), 61.93 (O-ethyl), 66.79 (O-benzyl), 128.01, 128.18, 128.43, 135.42, 167.68, 170.00, 170.74, 171.49. MS (CI, C_4H_9): m/z (%) = 459 (4) [M$^+$], 368 (100). Anal. ($C_{24}H_{33}N_3O_6$) C,H,N.

***N*-{[(2*R*,3*R*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucyl-(*S*)-proline Benzyl Ester (16b) (EtO-(*R*,*R*)-Azi-Leu-Pro-OBzl).** DPPA-mediated coupling of **15b** with Pro-OBzl HCl gave after purification by column chromatography (ethyl acetate, R_f = 0.56) **16b** (1.3 g, 57%) as a colorless viscous liquid: $[\alpha]_D^{21}$ = −125.0° (c 0.44, EtOH). IR (ethyl acetate): 3275 (br), 1742, 1656, 1539, 1448 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.88 (d, J = 6.6 Hz, 3 H), 0.92 (d, J = 6.6 Hz, 3 H), 1.26 (t, J = 7.1 Hz, 3 H), 1.4–1.52 (m, 2 H, β-H Leu), 1.58–1.7 (m, 1 H, γ-H Leu), 1.74 (bt, J = 8.0 Hz, 1 H, NH Azi), 1.9–2.1 (m, 3 H, β-H Pro, 2 γ-H Pro), 2.2 (m, 1 H, β-H Pro), 2.64 (d, J = 7.6 Hz, 1 H, Azi), 2.80 (d, J = 8.5 Hz, 1 H, Azi), 3.56 (m, 1 H, δ-H Pro), 3.64 (m, 1 H, δ-H Pro), 4.18 (q, J = 7.1 Hz, 2 H), 4.52 (m, 1 H, α-H Pro), 4.70 (m, 1 H, α-H Leu), 5.04 (d, J = 12.2 Hz, 1 H), 5.16 (d, J = 12.2 Hz, 1 H), 6.86 (bd, J = 8.0 Hz, 1 H, NH Leu), 7.2–7.5 (m, 5 H). ^{13}C NMR (CDCl$_3$): δ 14.14, 21.78, 23.40, 24.70, 24.99, 29.02, 35.58, 37.49, 41.48, 46.90, 48.40, 58.98, 62.19, 67.03, 128.25, 128.40, 128.63, 135.62, 167.38, 170.50, 170.92, 171.74. MS (EI 70 eV): m/z (%) = 459 (27) [M$^+$], 227 (100). Anal. ($C_{24}H_{33}N_3O_6$) C,H,N.

Diethyl (2*S*,3*S*)-1-[*N*-(Fluoren-9-ylmethoxycarbonyl)-(*S*)-leucyl]aziridine-2,3-dicarboxylate (20a) (Fmoc-Leu-(*S*,*S*)-Azi-(OEt)$_2$). N-Acylation of **10a** via symmetric anhydride using Fmoc-Leu yielded after column chromatography (cyclohexane/ethyl acetate, 5/1, R_f = 0.21) **20a** (1.6 g, 44%): mp 56 °C; $[\alpha]_D^{21.5}$ = −10.52° (c 1.085, EtOH). IR (CH$_2$Cl$_2$): 3418 (br), 1738, 1527 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.9 (d, J = 6.6 Hz, 6 H), 1.23 (t, J = 7.1 Hz, 3 H), 1.25 (t, J = 7.1 Hz, 3 H), 1.45–1.8 (m, 3 H), 3.5 (s, 2 H, Azi), 4.15–4.3 (m, 3 H, Fmoc), 4.4 (q, J = 7.1 Hz, 4 H), 5.15 (m, 1 H, α-H), 5.3 (bd, J = 8.0 Hz, 1 H, NH), 7.2–7.4 (m, 4 H), 7.5–7.6 (m, 2 H), 7.7–7.8 (m, 2 H). ^{13}C NMR (CDCl$_3$): δ 13.97, 21.74, 22.73, 24.66, 39.89, 41.44, 47.20, 54.37, 62.62, 66.98, 119.96, 125.11, 127.05, 127.68, 141.29, 143.74, 155.79, 166.06, 180.63. MS (EI, 70 eV): m/z (%) = 522 (10) [M$^+$], 178 (100). MALDI-TOF HRMS: calcd, 545.22637 [M + Na]$^+$; found, 545.2252 ± 0.00174. Anal. ($C_{28}H_{34}N_2O_7$) C,H; N: calcd, 5.36; found, 4.25.

***N*-{(2*S*,3*S*)-1-[[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]-3-(ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine Benzyl Ester (21a) (BOC-Phe-(EtO)-(*S*,*S*)-Azi-Leu-OBzl).** N-Acylation of **14a** (1.6 mmol, 579 mg) via the symmetric anhydride procedure using BOC–Phe gave, after column chromatography (cyclohexane/ethyl acetate, 1/1, R_f = 0.7), **21a** (347 mg, 95%): mp 50 °C; $[\alpha]_D^{27.3}$ = −10.6° (c 0.25, EtOH). IR (KBr): 3359 (br), 1743, 1710, 1488 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.9 (t, J = 5.3 Hz, 6 H), 1.3 (t, J = 7.3 Hz, 3 H), 1.4 (s, 9 H), 1.45–1.7 (m, 3 H, Leu), 2.6 (d, J = 1.95 Hz, 1 H, Azi), 3.0–3.2 (m, 2 H, β-H Phe), 3.28 (d, J = 1.95 Hz, 1 H, Azi), 4.23 (m, 2 H, OCH$_2$), 4.45 (m, 1 H, α-H Phe), 4.6 (m, 1 H, α-H Leu), 5.18 (s, 2 H), 5.28 (bd, J = 8.3 Hz, 1 H, NH Phe), 6.34 (bd, J = 8.1 Hz, 1 H, NH Leu), 7.12–7.28 (m, 5 H), 7.28–7.4 (m, 5 H). ^{13}C NMR (CDCl$_3$): δ 13.93 (CH$_3$), 21.85, 22.71 (Leu CH$_3$), 24.91 (Leu), 28.25 (BOC), 39.58 (Azi), 40.57 (Azi), 41.34 (double peak, β-C Leu, Phe), 50.88 (α-C Leu), 57.0 (α-C Phe), 62.63, 67.25 (OCH$_2$), 80.01 (BOC), 126.87, 128.22, 128.48, 128.54, 128.65, 129.54, 135.35, 136.73, 154.87, 164.76, 165.76, 171.93, 178.35. HREIMS (70 eV): calcd, 609.3050; found, 609.3051. Anal. ($C_{33}H_{43}N_3O_8$) C,H,N.

***N*-{(2*R*,3*R*)-1-[[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]-3-(ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine Benzyl Ester (21b) (BOC-Phe-(EtO)-(*R*,*R*)-Azi-Leu-OBzl).** N-Acylation of **14b** (1.0 mmol, 362 mg) via the symmetric anhydride procedure using BOC-Phe gave, after column chromatography (cyclohexane/ethyl acetate, 1/1, R_f = 0.7), **21b** (429 mg, 70%) as a colorless viscous liquid: $[\alpha]_D^{24.4}$ = −34.7° (c 0.36, EtOH). IR (KBr): 3339 (br), 1742, 1498 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.9 (d, J = 6.1 Hz, 6 H), 1.28 (t, J = 7.1 Hz, 3 H), 1.38 (s, 9 H), 1.44–1.66 (m, 3 H, Leu), 3.1–3.26 (m, 2 H, β-H Phe), 3.25 (d, J = 2.2 Hz, 1 H, Azi), 3.51 (d, J = 2.2 Hz, 1 H, Azi), 4.22 (q, J = 7.1 Hz, 2 H), 4.44–4.66 (m, 2 H, α-H Leu, Phe), 4.92 (bd, J = 9.0 Hz, 1 H, NH Phe), 5.16 (s, 2 H), 6.4 (bd, J = 8.0 Hz, 1 H, NH Leu), 7.15–7.4 (m, 10 H). ^{13}C NMR (CDCl$_3$): δ 13.88, 21.75, 22.66, 24.75, 28.15, 38.22, 40.94, 41.19, 41.37, 50.84, 56.16, 62.50, 67.16, 80.09, 126.81, 128.15, 128.44, 128.57, 129.48, 135.14, 136.11, 154.87, 164.93, 165.81, 172.02, 179.26. MS (EI, 70 eV): m/z (%) = 609 (31) [M$^+$], 418 (100). Anal. ($C_{33}H_{43}N_3O_8$) C,H,N.

***N*-{(2*S*,3*S*)-1-[[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]-3-(ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucyl-

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(S)-proline Benzyl Ester (22a) (BOC-Phe-(EtO)-(S,S)-Azi-Leu-Pro-OBzl). N-Acylation of 16a (1.5 mmol, 688 mg) with BOC-Phe via the symmetric anhydride method gave, after purification by flash chromatography (cyclohexane/ethyl acetate, 1/1, R_f = 0.6), 560 mg (53%) of 22a: mp 80 °C; $[\alpha]_D^{23.1}$ = −46.7° (c 0.51, EtOH). IR (KBr): 3275 (br), 1744, 1713, 1632, 1498, 1456 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.88 (d, J = 6.35 Hz, 3 H, δ-H Leu), 0.92 (d, J = 6.34 Hz, 3 H, δ-H Leu), 1.24 (t, J = 7.1 Hz, 3 H), 1.35 (s, 9 H, BOC), 1.42−1.8 (m, 3 H, β-H, γ-H Leu), 1.85−2.1 (m, 3 H, 1 β-H, 2 γ-H Pro), 2.1−2.3 (m, 1 H, β-H, Pro), 2.94−3.28 (m, 2 H, β-H Phe), 3.35 (d, J = 2.0 Hz, 1 H, Azi), 3.45 (d, J = 2.0 Hz, 1 H, Azi), 3.52−3.64 (m, 1 H, δ-H, Pro), 3.66−3.80 (m, 1 H, δ-H, Pro), 4.12−4.24 (m, 2 H, OCH$_2$), 4.4 (m, 1 H, α-H Phe), 4.58 (m, 1 H, α-H Pro), 4.78 (m, 1 H, α-H Leu), 5.08 (d, J = 12.2 Hz, 1 H), 5.20 (d, J = 12.2 Hz, 1 H), 5.36 (bd, J = 7.81, 1 H, NH Phe), 6.81 (bd, J = 8.7 Hz, 1 H, NH Leu), 7.2−7.4 (m, 10 H). ^{13}C NMR (CDCl$_3$): δ 13.9 (OCH$_2$CH$_3$), 21.61, 23.23 (δ-C Leu), 24.58 (γ-C Pro), 24.79 (γ-C Leu), 28.19 (BOC), 28.90 (β-C Pro), 39.67 (β-C Phe), 40.06 (Azi), 40.72 (Azi), 41.57 (β-C Leu), 46.83 (δ-C Pro), 48.86 (α-C Leu), 57.11 (α-C Phe), 58.92 (α-C Pro), 62.61 (OCH$_2$), 66.91 (OCH$_2$-Ph), 79.48 (BOC), 126.67, 128.10, 128.30, 128.42, 128.52, 129.65, 135.47 (double peak), 154.71, 164.62, 165.97, 170.57, 171.51, 179.35. MS (CI, C$_4$H$_8$): m/z (%) = 706 (100) [M$^+$]. Anal. ($C_{38}H_{50}N_4O_9$) C,H; N: calcd, 7.93; found, 7.46.

***N*-{(2R,3R)-1-{[N-(tert-Butoxycarbonyl)-(S)-phenylalanyl]-3-(ethoxycarbonyl)aziridin-2-yl]carbonyl}-(S)-leucyl-(S)-proline Benzyl Ester (22b) (BOC-Phe-(EtO)-(R,R)-Azi-Leu-Pro-OBzl).** N-Acylation of 16b (0.15 mmol, 69 mg) with BOC-Phe via the symmetric anhydride method gave, after purification by flash chromatography (cyclohexane/ethyl acetate, 1/1, R_f = 0.7), 60 mg (57%) of 22b: mp 77 °C; $[\alpha]_D^{24.7}$ = −61.4° (c 0.3, EtOH). IR (CH$_2$Cl$_2$): 3418 (br), 1742, 1641 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.9 (d, J = 6.6 Hz, 3 H), 0.96 (d, J = 6.6 Hz, 3 H), 1.26 (t, J = 7.1 Hz, 3 H), 1.36 (s, 9 H), 1.44−1.76 (m, 3 H, Leu), 1.88−2.1 (m, 3 H, Pro), 2.1−2.3 (m, 1 H, Pro), 3.0−3.24 (m, 2 H, Phe), 3.4 (d, J = 1.96, 1 H, Azi), 3.47 (d, J = 1.96 Hz, Azi), 3.53 (m, 1 H, Pro), 3.76 (m, 1 H, Pro), 4.2 (q, J = 7.1 Hz, 2 H), 4.50−4.66 (m, 2 H, α-H Phe, Pro), 4.74 (m, 1 H, α-H Leu), 5.0 (bd, J = 9.0 Hz, 1 H, NH Phe), 5.09 (d, J = 12.2 Hz, 1 H), 5.2 (d, J = 12.2 Hz, 1 H), 6.7 (bd, J = 8.0 Hz, 1 H, NH), 7.14−7.4 (m, 10 H). ^{13}C NMR (CDCl$_3$): δ 13.87, 21.68, 23.26, 24.65, 24.83, 28.16, 28.89, 38.42, 41.18, 41.31, 41.42, 46.81, 49.12, 56.25, 58.89, 62.50, 66.90, 79.89, 126.82, 128.10, 128.30, 128.43, 128.52, 129.66, 135.46, 136.13, 154.78, 164.88, 165.92, 170.57, 171.61, 179.52. MS (EI, 70 eV): m/z (%) = 706 (46) [M$^+$], 206 (100). Anal. ($C_{38}H_{50}N_4O_9$) C,H; N: calcd, 7.93; found, 7.40.

(2S,3S)- and (2R,3R)-2-Benzyl 3-Ethyl 1-[N-(tert-Butoxycarbonyl)-(S)-phenylalanyl]aziridine-2,3-dicarboxylate (23a+b) (BOC-Phe-(S,S+R,R)-Azi-(OEt)(OBzl). N-Acylation of 13a+b (2 mmol, 498 mg) with BOC−Phe via the symmetric anhydride procedure gave, after column chromatography (cyclohexane/ethyl acetate, 2/1, R_f = 0.65) 23a+b (23a (S,S)/23b (R,R) = 5/4) (850 mg, 86%) as a yellowish viscous liquid: $[\alpha]_D^{22.7}$ = −10.2° (c 0.71, EtOH). IR (ethyl acetate): 3377 (br), 1743, 1712, 1497, 1455 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 1.28 (t, J = 7.1 Hz, 3 H), 1.36 and 1.38 (s, together 9 H), 3.0−3.3 (m, 2 H, β-H Phe), 3.35 (S,S) and 3.49 (R,R) (d, J = 2.2 Hz, together 1 H, CH Azi), 3.40 (S,S) and 3.53 (R,R) (d, J = 2.2 Hz, together 1 H, CH Azi), 4.22 (q, J = 7.1 Hz, 2 H), 4.5 (S,S) and 4.62 (R,R) (m, together 1 H, α-H Phe), 4.93 (R,R) and 5.1 (S,S) (bd, J = 9.0 Hz, together 1 H, NH), 5.2 (m, 2 H), 7.2−7.45 (m, 10 H). ^{13}C NMR (CDCl$_3$): δ 13.75, 28.02, 28.06 (BOC), 38.09, 39.08, 39.61, 39.87, 39.93, 40.21 (β-C Phe, Azi), 55.97, 56.37 (α-C Phe), 62.31, 62.36, 67.91, 67.93 (OCH$_2$), 79.48, 79.64 (BOC), 126.57, 126.60, 128.14, 128.22, 128.30, 128.40, 128.43, 128.48, 128.52, 128.58, 129.43, 134.38, 134.41, 136.01, 136.11, 154.60, 154.75, 165.67, 165.74, 178.78, 178.89. MS (CI, C$_4$H$_8$): m/z (%) = 496 (75) [M$^+$], 405 (100). Anal. ($C_{27}H_{32}N_2O_7$) C,H,N.

Diethyl (2S,3S)-1-[N-(Benzyloxycarbonyl)glycylglycyl]aziridine-2,3-dicarboxylate (24a) (Z-Gly-Gly-(S,S)-Azi-(OEt)$_2$. N-Acylation of 10a (1.5 mmol, 280 mg) with Z-Gly-Gly by the general EEDQ procedure gave after column chromatography (ethyl acetate, R_f = 0.64) 24a (320 mg, 49%) as a colorless viscous liquid: $[\alpha]_D^{24.5}$ = +15.8° (c 0.92, EtOH). IR (CH$_2$Cl$_2$): 3395 (br), 1737, 1532 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 1.2 (t, J = 7.1 Hz, 6 H), 3.38 (s, 2 H, Azi), 3.8−4.08 (m, 4 H, Gly), 4.15 (q, J = 7.1 Hz, 4 H), 5.02 (s, 2 H), 6.72 (bt, J = 5.8 Hz, 1 H, NH), 6.95 (bs, 1 H, NH), 7.25 (m, 5 H). ^{13}C NMR (CDCl$_3$): δ 13.93, 39.72, 43.71, 44.32, 62.77, 67.20, 128.06, 128.17, 128.50, 136.09, 156.52, 166.00, 169.06, 176.54. MS (EI, 70 eV): m/z (%) = 435 (28) [M$^+$], 188 (100). MALDI-TOF HRMS: calcd, 458.1539 [M + Na]$^+$; found, 458.16082 ± 0.00637. Anal. ($C_{20}H_{25}N_3O_8$) H,N; C: calcd, 55.17; found, 54.23.

Diethyl (2S,3S)-1-[N-(Benzyloxycarbonyl)-(S)-alanyl-(S)-phenylalanyl]aziridine-2,3-dicarboxylate (25a) (Z-Ala-Phe-(S,S)-Azi-(OEt)$_2$. N-Acylation of 10a (5 mmol, 936 mg) with Z-Ala-Phe by the general EEDQ procedure gave, after column chromatography (cyclohexane/ethyl acetate, 2/1, R_f = 0.14), 25a (1.17 g, 42.8%) as a yellowish viscous liquid: $[\alpha]_D^{23.1}$ = +9.2 (c 0.82, EtOH). IR (CH$_2$Cl$_2$): 3423 (br), 1738, 1523 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 1.22 (d, J = 6.6 Hz, 3 H, Ala), 1.28 (t, J = 7.1 Hz, 6 H), 3.06−3.34 (m, 2 H, Phe), 3.5 (s, 2 H, Azi), 4.18−4.3 (m, 5 H, α-H Ala, 2 OCH$_2$), 4.9−5.02 (m, 1 H, α-H Phe), 5.08 (d, J = 12.2 Hz, 1 H), 5.16 (d, J = 12.2 Hz, 1 H), 5.26 (bs, 1 H, NH Ala), 6.44 (bd, J = 8.7 Hz, 1 H, NH Phe), 7.16−7.4 (m, 10 H). ^{13}C NMR (CDCl$_3$): δ 13.91, 18.61, 37.77, 40.25, 50.48, 54.39, 62.56, 67.02, 126.98, 128.01, 128.43, 128.48, 129.52, 135.73, 136.12, 155.74, 165.87, 171.70, 178.46. MS (EI, 70 eV): m/z (%) = 539 (63) [M$^+$], 91 (100). Anal. ($C_{27}H_{31}N_3O_8$) C,H,N.

Diethyl (2R,3R)-1-[N-(Benzyloxycarbonyl)-(S)-alanyl-(S)-phenylalanyl]aziridine-2,3-dicarboxylate (25b) (Z-Ala-Phe-(R,R)-Azi-(OEt)$_2$). N-Acylation of 10b (3.3 mmol, 617 mg) with Z-Ala-Phe by the general EEDQ procedure gave, after purification by column chromatography (cyclohexane/ethyl acetate, 3/1, R_f = 0.16), 25b (410 mg, 23.1%): mp 57 °C; $[\alpha]_D^{25}$ = −35.78° (c 1.034, EtOH). IR (ethyl acetate): 3326 (Br), 1738, 1675, 1520, 1455 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 1.26 (d, J = 6.6 Hz, 3 H, Ala), 1.27 (t, J = 7.1 Hz, 6 H), 3.0−3.35 (m, 2 H, Phe), 3.5 (s, 2 H, Azi), 4.16−4.25 (m, 5 H, α-H Ala, 2 OCH$_2$), 4.90−5.0 (m, 1 H, α-H Phe), 5.05−5.2 (m, 3 H, OCH$_2$, NH Ala), 6.6 (bd, J = 8.1 Hz, 1 H, NH Phe), 7.1−7.4 (m, 10 H). ^{13}C NMR (CDCl$_3$): δ 13.88, 18.16, 37.81, 40.27, 50.58, 54.44, 62.58, 67.19, 127.00, 128.17, 128.28, 128.46, 128.57, 129.54, 135.73, 136.07, 155.80, 165.83, 171.56, 178.32. MS (CI, C$_4$H$_{10}$): m/z (%) = 540 (5) [M$^+$ + 1], 353 (100). Anal. ($C_{27}H_{31}N_3O_8$): C,H,N.

Diethyl (2S,3S)-1-[N-(tert-Butoxycarbonyl)-(S)-phenylalanyl-(S)-alanyl]aziridine-2,3-dicarboxylate (26a) (BOC-Phe-Ala-(S,S)-Azi-(OEt)$_2$). N-Acylation of 10a (1.5 mmol, 280 mg) with BOC-Phe-Ala by the general EEDQ method yielded, after purification by column chromatography (cyclohexane/ethyl acetate, 3/1, R_f = 0.15), 26a (580 mg, 77%): mp 89 °C; $[\alpha]_D^{23.3}$ = −4.7° (c 0.48, EtOH). IR (ethyl acetate): 3282 (br), 1734, 1446 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 1.26 (t, J = 7.1 Hz, 6 H), 1.35 (s, 9 H, BOC), 1.4 (d, J = 6.6 Hz, 3 H, Ala), 2.9−3.1 (m, 2 H, Phe), 3.4 (s, 2 H, Azi), 4.1−4.38 (m, 5 H, α-H Phe, 2 OCH$_2$), 4.5 (m, 1 H, α-H Ala), 4.96 (bs, 1 H, NH Phe), 6.7 (bd, J = 7.8 Hz, 1 H, NH Ala), 7.1−7.3 (m, 5 H). ^{13}C NMR (CDCl$_3$): δ 13.98 (OCH$_2$CH$_3$), 17.92 (β-C Ala), 28.15 (BOC), 39.75 (β-C Phe), 40.03 (Azi), 49.23 (α-C), 49.94 (α-C), 62.66 (OCH$_2$), 80.0 (BOC), 126.76, 128.47, 129.35, 136.54, 156.5 (BOC), 165.93, 170.34, 179.96. MS (CI, C$_4$H$_8$): m/z (%) = 506 (100) [M$^+$]. Anal. ($C_{25}H_{35}N_3O_8$) C,H; N: calcd, 8.31; found, 7.42.

(2S,3S)- and (2R,3R)-2-Benzyl 3-Ethyl 1-[N-(tert-Butoxycarbonyl)-(S)-phenylalanyl-(S)-alanyl]aziridine-2,3-dicarboxylate (27a+b) (BOC-Phe-Ala-(S,S+R,R)-Azi-(OEt)-(OBzl)). N-Acylation of 13a+b (0.4 mmol, 100 mg) with BOC-Phe-Ala by the general EEDQ procedure gave, after column chromatography (cyclohexane/ethyl acetate, 3/1, R_f = 0.13), 27a+b (150 mg, 66%) (1.3/1): mp 115 °C; $[\alpha]_D^{21.3}$ = −11.4° (c 0.37, EtOH). IR (ethyl acetate): 3305 (br), 1741, 1720, 1664, 1524, 1500, 1455 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 1.2−1.45 (m, 16 H), 3.0−3.15 (m, 2 H), 3.4−3.55 (m, 2 H), 4.15−4.3 (m, 3 H, α-H Phe, OCH$_2$), 4.55 and 4.7 (m, together 1 H, α-H Ala), 4.9 (bs, 1 H, NH Phe), 5.2 (s, 2 H), 6.55 and 6.65 (bs, together 1

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H, NH Ala), 7.1–7.3 (m, 10 H). ^{13}C NMR (CDCl$_3$): δ 13.9, 18.18, 26.90, 28.21 (BOC), 39.70, 39.99 (β-C Phe), 40.05, 40.23 (Azi), 49.61, 50.01 (α-C Phe), 62.64, 62.72 (OCH$_2$), 68.16, 68.20 (OCH$_2$Ph), 80.0 (BOC), 126.97, 128.63, 128.68, 128.72, 128.74, 128.81, 129.14, 129.37, 129.43, 134.46, 134.55, 136.35, 136.44, 156.0, 165.78, 165.82, 165.88, 165.95, 170.36, 170.61, 179.26, 179.96. MS (CI, C$_4$H$_{10}$): *m/z* (%) = 567 (62) [M$^+$], 476 (100). MALDI-TOF HRMS: calcd, 590.2478 [M + Na]$^+$; found, 590.2464 ± 0.0010. Anal. ($C_{30}H_{37}N_3O_8$) H,N; C: calcd, 63.48; found, 64.15.

(2*S*,3*S*)- and (2*R*,3*R*)-2-Benzyl 3-Ethyl 1-[*N*-(*tert*-Butoxycarbonyl)-(*S*)-leucylglycyl]aziridine-2,3-dicarboxylate (28a+b) (BOC-Leu-Gly-(*S*,*S*+*R*,*R*)-Azi-(OEt)(OBzl)). N-Acylation of 13a+b (1.6 mmol, 398 mg) with BOC-Leu-Gly via the EEDQ method gave, after flash chromatography (cyclohexane/ethyl acetate, 3/1, R_f = 0.14), 28a+b (1.5/1) (430 mg, 51.7%): mp 88 °C; $[\alpha]_D^{24.2}$ = −18.3° (*c* 0.59, EtOH). IR (ethyl acetate): 3338 (br), 1741, 1515, 1455 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 0.9 (d, *J* = 6.6 Hz, 3 H), 0.92 (d, *J* = 6.6 Hz, 3 H), 1.25 und 1.28 (t, *J* = 7.1 Hz, together 3 H), 1.45 (m, 11 H, BOC, β-H Leu), 1.6–1.8 (m, 1 H, γ-H Leu), 3.45–3.55 (m, 2 H, Azi), 3.95–4.10 (m, 1 H, 1 α-H Gly), 4.15–4.35 (m, 4 H, α-H Leu, 1 α-H Gly, OCH$_2$), 4.9 (bs, 1 H, NH Leu), 5.2 (s, 2 H), 6.7 (bs, 1 H, NH Gly), 7.2–7.4 (m, 5 H). ^{13}C NMR (CDCl$_3$): δ 13.94, 14.08 (OCH$_2$CH$_3$), 21.74, 22.97 (CH$_3$ Leu), 24.70 (γ-C Leu), 28.26 (BOC), 39.63, 39.84 (Azi), 41.28 (β-C Leu), 43.77 (α-C Gly), 53.02 (α-C Leu), 61.43, 62.73 (OCH$_2$), 68.31 (OCH$_2$-Ph), 80.0 (BOC), 126.98, 128.64, 128.72, 128.88, 129.12, 130.99, 134.36, 156.0 (BOC), 165.87 (double peak), 172.57, 176.46. MS (CI, C$_4$H$_9$): *m/z* (%) = 519 [M$^+$], 428 (100). Anal. ($C_{26}H_{37}N_3O_8$) C,H; N: calcd, 8.09; found, 7.64.

Diethyl (2*S*,3*S*)-1-[*N*-(Benzyloxycarbonyl)glycyl-(*S*)-phenylalanyl-(*S*)-alanyl]aziridine-2,3-dicarboxylate (29a) (Z-Gly-Phe-Ala-(*S*,*S*)-Azi-(OEt)$_2$). N-Acylation of 10a (0.4 mmol, 74 mg) with Z-Gly-Phe-Ala using the general EEDQ procedure gave, after purification by column chromatography (cyclohexan/ethyl acetate, 3/1, R_f = 0.1), 29a (110 mg, 46%): mp 89 °C; $[\alpha]_D^{24.6}$ = +6.3° (*c* 0.5, EtOH). IR (ethyl acetate): 3305 (br), 1738, 1656, 1455 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 1.0–1.4 (m, 9 H, β-H Ala, 2 CH$_3$), 3.08 (m, 2 H, β-H Phe), 3.45 (s, 2 H, Azi), 3.75–4.0 (m, 2 H, Gly), 4.2 (q, *J* = 7.1 Hz, 4 H, OCH$_2$), 4.6 (m, 1 H, α-H Ala), 4.8 (m, 1 H, α-H Phe), 5.1 (s, 2 H, OCH$_2$Ph), 5.7 (bs, 1 H, NH Gly), 6.75 (bd, *J* = 8.8 Hz, 1 H, NH Phe), 6.88 (bs, 1 H, NH Ala), 7.1–7.5 (m, 10 H). ^{13}C NMR (CDCl$_3$): δ 13.92 (CH$_2$CH$_3$), 17.84 (CH$_3$ Ala), 38.24 (β-C Phe), 40.10 (Azi), 44.66 (Gly), 49.41 (α-C Ala), 54.25 (α-C Phe), 62.56 (OCH$_2$), 67.22 (OCH$_2$Phe), 127.02, 128.08, 128.20, 128.50, 128.63, 129.29, 138.11, 136.23, 155.0, 166.08, 169.27, 170.02, 179.36. MS (CI, C$_4$H$_{10}$): *m/z* (%) = 596 (93) [M$^+$], 488 (100). Anal. ($C_{30}H_{36}N_4O_9$) C,H,N.

(2*S*,3*S*)- and (2*R*,3*R*)-1-[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]-3-(ethoxycarbonyl)aziridine-2-carboxylic Acid (30a+b) (BOC-Phe-(*S*,*S*+*R*,*R*)-Azi-(OEt)(OH)). Catalytic hydrogenolysis of 23a+b by the general method described above gave, after recrystallization from acetonitrile, 30a+b (1/1) (234 mg, 96%): mp 83 °C; $[\alpha]_D^{23.1}$ = −1.0° (*c* 0.49, EtOH). IR (ethyl acetate): 3376 (br), 1748, 1720, 1497, 1455 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 1.1–1.5 (m, 12 H), 3.1 (m) and 3.3 (m) (together 2 H, β-H Phe), 3.4–3.6 (m, 2 H, Azi), 4.23 (q, *J* = 7.1 Hz, 2 H), 4.4 (m) and 4.6 (m) (together 1 H, α-H Phe), 5.15 (bd, *J* = 8.8 Hz) and 5.3 (bd, *J* = 8.9 Hz) (together 1 H, NH), 7.0–7.4 (m, 5 H), 9.5 (bs, 1 H, COOH). ^{13}C NMR (CDCl$_3$): δ 13.81 (CH$_3$), 27.98, 28.05 (BOC), 38.31 (double peak), 38.83, 39.19, 39.61, 40.66 (β-C Phe, Azi), 56.18 (α-C Phe), 62.17, 62.25 (OCH$_2$), 80.31 (BOC), 126.64, 126.83, 128.26, 129.09, 129.19, 129.41, 135.79, 135.99, 156.19 (double peak), 166.14 (double peak), 169.00 (double peak), 178.86, 179.60. MS (CI, C$_4$H$_{10}$): *m/z* (%) = 463 (4) [M$^+$ + C$_4$H$_9$], 407 (46) [M$^+$ + 1], 351 (100). Anal. ($C_{19}H_{24}N_2O_7$) H,N; C: calcd, 59.10; found, 60.30.

Kinetic Measurements. Kinetic measurements were carried out on a Pharmacia LKB Ultrospec III photometer and on a Perkin-Elmer LS-3B fluorescence spectrometer, respectively. The following assay buffers were used: papain/L-BAPA,[53] 50 mM phosphate buffer, pH 6.5, 5 mM EDTA, 5 mM cysteine; papain/Z-Phe-Arg-AMC[54] and cathepsin H/H-Arg-AMC,[55] 50 mM phosphate buffer, pH 6.5, 2.5 mM EDTA, 2.5 mM DTT, 0.005% Brij 35; cathepsin B[56] or cathepsin L/Z-Phe-Arg-AMC,[55–57] 50 mM citrate buffer, pH 6.0, 2.5 mM DTT, 5 mM EDTA, 200 mM NaCl, 0.005% Brij 35; calpain I or II/Suc-Leu-Tyr-AMC,[58] 50 mM TRIS buffer, pH 7.5, 5 mM CaCl$_2$, 2.5 mM EDTA, 2.5 mM DTT; trypsin/L-BAPA[59] or chymotrypsin/Ac-Phe-pNA,[60] 0.1 M phosphate buffer, pH 7.6; elastase/Ac-Ala-Ala-Ala-pNA,[61] 0.1 M TRIS buffer, pH 8.0; thermolysin/FAGLA,[62] 0.1 M TRIS buffer, pH 7.22, 10 mM CaCl$_2$; pepsin/Ac-Phe-3,5-diiodo-TyrOH,[63] 0.22 mM acetic acid. Assay buffers for the inactivation of papain at different pH values were 50 mM citrate for pH 4–6, phosphate for pH 6–8, and borate for pH 8–9. All kinetic experiments were done at 25 °C. Enzyme solutions were prepared fresh by incubating the enzymes in the individual reaction buffers at 25 °C for 30 min. Substrates and inhibitors were dissolved in DMSO, the final concentrations at measurements not being higher than 12% DMSO. Substrate solutions were prepared by diluting a stock solution with buffer, and inhibitor solutions were diluted with water. Substrate hydrolysis in assays using fluorogenic substrates was monitored by the increase of fluorescence at 460 nm (irradiation at 380 nm) and in assays using nitroanilide substrates by absorption increase at 405 nm. Thermolysin was assayed by absorption decrease at 345 nm. Pepsin was assayed by absorption increase at 570 nm. The inactivation rates (k_{obs}) for different inhibitor concentrations in the presence of the substrate were determined according to the continuous method of Tian and Tsou[64] by monitoring the product released from hydrolysis of the substrate in the presence of the inhibitor as a function of time (fluorescence or absorption = $A(1 - \exp(-k_{obs}t)) + B$) until complete inactivation of the enzyme (typically 5–60 min), with steady-state conditions established during inactivation time. This was done at constant enzyme and various (3–7) inhibitor concentrations, respectively. For weak inhibitors where complete inactivation took longer than 60 min, k_{obs} values were determined by the dilution assay of Kitz and Wilson.[65] Thereby enzyme and inhibitor were incubated. After an incubation time of 5–60 min (5–7 values), the incubation mixture was diluted by adding substrate and buffer and the residual enzyme activity [E] was measured. k_{obs} values were then calculated using the equation $[E] = [E_0] \exp(-k_{obs}t)$. These experiments were repeated for 3–7 inhibitor concentrations. Fitting of the k_{obs} values, obtained by either continuous or dilution assays, against the inhibitor concentrations to the hyperbolic equation $k_{obs} = k_i[I]/K_i^{app} + [I]$ gave the individual values of K_i^{app} and k_i. The K_i^{app} values were corrected to zero substrate concentration by the term $1 + [S]/K_m$ in the equation $K_i = K_i^{app}/(1 + [S]/K_m)$. The second-order rate constants $k_{2nd} = k_i/K_i$ were directly calculated from the individual constants. In cases where no saturation kinetics were achieved ($[I] < K_i$) due to limited solubility of the inhibitors or high K_i values, the second-order rate constants were calculated from linear regression to the equation $k_{obs} = k_{app}[I]$ and corrected to zero substrate concentration from the equation $k_{2nd} = k_{app}(1 + [S]/K_m)$. K_i values for the non-time-dependent inhibition of cathepsin H and calpains I and II were obtained by Dixon plots[66] using the equation $v_0/v_i = 1 + [I]/K_i^{app}$ and corrected to zero substrate concentration from $K_i = K_i^{app}/(1 + [S]/K_m)$. The following K_m values were used: papain/Z-Phe-Arg-AMC 0.09 mM, papain/L-BAPA 2.5 mM, cathepsin B/Z-Phe-Arg-AMC 0.15 mM, cathepsin L/Z-Phe-Arg-AMC 6.5 μM, cathepsin H/H-Arg-AMC 0.15 mM, calpain I/Suc-Leu-Tyr-AMC 0.4 mM, calpain II/Suc-Leu-Tyr-AMC 0.22 mM. Enzyme concentrations were: papain 0.48 μg mL^{-1}, cathepsin B 0.015 μg mL^{-1}, cathepsin L 0.05 μg mL^{-1}, cathepsin H 0.082 μg mL^{-1}, calpain I 3.8 μg mL^{-1}, calpain II 0.04 mg mL^{-1}, elastase 7.5 μg mL^{-1}, chymotrypsin 0.15 mg mL^{-1}, trypsin 3 μg mL^{-1}, thermolysin 6.9 μg mL^{-1}, pepsin 0.6 mg mL^{-1}. The kinetic constants were obtained by nonlinear or linear regression analysis using the program GraFit.[67]

Dialysis Experiments. Dialysis experiments were carried out as follows. Papain (0.52 mg mL^{-1}) was inactivated by 17a (0.37 mM) and 11a (0.13 mM), respectively (60 min incubation time, each) and then subjected to dialysis (visking dialysis tubing type 27/32, Serva) against reaction buffer (60 min, each). No recovery of enzyme activity (substrate: L-BAPA, 1.18 mM) was detected, whereas control enzymes maintained 70%

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and 92% activity, respectively. Cathepsin L ($0.07\ \mu g\ mL^{-1}$) was inactivated by **28a+b** ($50\ \mu M$) (60 min incubation time) and then subjected to dialysis against reaction buffer (60 min). No recovery of enzymatic activity was detected, whereas the control enzyme maintained 52% activity.

Stoichiometry of Inactivation of Papain by 11a. A papain solution ($2.76\ \mu M$, as determined by active site titration with E-64 as described in ref 55) was titrated with **11a** (three values, substrate: L-BAPA, 1.18 mM, pH 6.6) by adding increasing amounts of the inhibitor solution ($[I] = 0.528\ \mu M$–1.056 mM) to the papain solution. After each aliquot of inhibitor was added, residual enzyme activity [E] was monitored. The intercept with the *x*-axis of a plot of [E] vs [I] gave the active site concentration; 1.11 ± 0.4 equiv of **11a** was required to totally inactivate 1 equiv of papain.

Standard inhibitors were *N*-ethylmaleimide[71] for the dilution assay and E-64[72] for the continuous assay, respectively.

Abbreviations. Amino acids are written in the three-letter codes and are L configured. Others: Azi (aziridine-2,3-dicarboxylic acid), Eps (epoxysuccinic acid), E-64 (1-[*N*-[(L-3-*trans*-carboxyoxiran-2-yl)carbonyl]-L-leucyl]amino]-4-guanidinobutane), DPSI (diphenyl sulfimide), EEDQ (1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline), DPPA (diphenyl phosphorazidate), DCC (dicyclohexylcarbodiimide), DMAP (4-(dimethylamino)pyridine), PLE (pig liver esterase), CCL (*Candida Cylindracea* lipase), PPL (porcine pancreatic lipase), LPR (lipase *Penicillium roquefortii*), LRA (lipase *Rhizopus arrhizus*), LAN (lipase *Aspergillus niger*), BPACK ((benzyloxycarbonyl)phenylalanyl-alanine chloromethyl ketone), BAPA (benzoylarginyl *p*-nitroanilide), AMC ((aminomethyl)coumarin), pNA (*p*-nitroanilide), Tris (tris(hydroxymethyl)aminomethane), FAGLA (3-(2-furyl)acryloyl-glycyl-leucine amide), DTT (dithiothreitol), EDTA (ethylenediamintetraacetic acid), Suc (succinyl).

Acknowledgment. Financial support of this work by the Deutschen Forschungsgemeinschaft DFG and the Faculty of Chemistry and Pharmacy, University of Freiburg, Germany, is gratefully acknowledged. I would like to thank Prof. J. Schultz, Department of Pharmaceutical Chemistry, University of Tuebingen, Germany, for a gift of cathepsin L; Dr. Beck and Dr. Jendralla, Fa. Hoechst AG, Frankfurt, Germany, for chromatographic purification of compounds **10a,b**, respectively, in large scale; and Dr. Waidelich, Perseptive Biosystems, Wiesbaden, Germany, for HR mass spectroscopy of several compounds.

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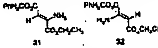
(70) Main product (2.5 g, 50%) of this reaction is a 2/1 mixture of enamines 31 and 32, isolated by column chromatography (cyclohexane/ethyl acetate, 5/1, R_f = 0.5). IR (ethyl acetate): 3485 (br), 3350 (br), 3034 (br), 1729, 1678, 1621, 1556, 1498, 1455 cm^{-1}. ^1H NMR ($CDCl_3$, 50 °C): δ 1.3 (t, J = 7.1 Hz, 32) and t (1.35, J = 7.1 Hz, 31) (together 3 H), 4.2 (q, J = 7.1 Hz, 32) and 4.25 (q, J = 7.1 Hz, 31) (together 2 H), 5.2 (s, 31) and 5.3 (s, 32) (together 2 H), 5.58 (s, 32) and 5.61 (s, 31) (together 1 H), 6.5 (bs, 2 H), 7.2–7.45 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 14.23 (31), 14.03 (32) (CH_3), 50.76 (32), 62.66 (31) (OCH_2), 66.62 (31), 68.20 (32) (OCH_2Ph), 85.81 (31), 89.45 (32) (HC=C), 128.25, 128.41, 128.60, 128.76, 128.84, 130.93, 135.10, 136.62, 146.38 (32), 147.03 (31) (HC=C), 163.80 (31), 163.84 (32), 169.86 (31), 170.05 (32). Anal. ($C_{13}H_{15}NO_4$) C,H,N. Assignment of NMR signals was carried out by INEPT long-range NMR spectroscopy.



31 32

(71) A k_{2nd} = 149 ± 19 M^{-1} min^{-1} was found for inhibition of papain by N-ethylmaleimide (Anderson, B.; Vasini, E. *Biochemistry* 1970, *9*, 2348–2352: k_{2nd} = 163 M^{-1} min^{-1}).
(72) A k_{2nd} = 8.4 ± 0.5 × 10^7 M^{-1} min^{-1} was found for inhibition of papain by E-64 (ref 1: 2.2–3.6 × 10^7 M^{-1} min^{-1}).
(73) The following equation was used:[67] k_{2nd} = (limit × $10^{(pH - pK_{a1})}$)/($10^{(2pH - pK_{a1} - pK_{a2})}$ + $10^{(pH - pK_{a1})}$ + 1). The following values were found for 11a: pK_{a1} = 4.8 ± 0.2, pK_{a2} = 3.8 ± 0.2, limit = 6043 M^{-1} s^{-1}.
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(76) Structures have been minimized using the Systematic Search program from Sybyl 6.4 (Tripos Associates Inc., St. Louis, MO, 1997). Energy minimization: Tripos force field, convergence criterion 0.001 kcal mol^{-1}. The superposition has been performed using the program Multifit from Sybyl 6.4.
(77) The docking of 28a into the active site of papain has been performed using the program FlexiDock from the Sybyl 6.4 Biopolymer Module (Tripos Associates Inc., St. Louis, MO). Energy minimization: Tripos force field, Gasteiger-Hueckel charges on ligand, Kollmann charges on protein. As FlexiDock pocket a region of 0.4 nm around the amino acids Cys25, Tyr67, Pro68, Trp69, Val133, Val157, His159, Ala160, and Phe207 of papain has been defined. The structure of papain has been taken from the Brookhaven Protein Databank (entry 1pe6).

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EXHIBIT C

DRAFT – 12/11/2001

Docket No. JBP 438

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Seiberg, et al.	
Serial No.	:	09/206,249	Art Unit: 1651
Filed	:	December 7, 1998	Examiner: M. Meller
For	:	METHOD FOR REGULATING PHAGOCYTOSIS	

DECLARATION OF KATHARINE MARTIN

I, Katharine Martin, hereby declare:

1. I am currently employed by Johnson & Johnson Consumer Companies, Inc. in the capacity of Manager, Pharmacology. I began employment with Johnson & Johnson Consumer Products, Inc. in 1990 as Scientist, *In Vitro* Toxicology.

2. I received a Bachelors of Science degree from the University of Bath in England. I am knowledgeable in the area of protein activity due to my experience in Biological Sciences.

3. Prior to 1986, it was well-known to those skilled in the art of protein biochemistry that the conformation of proteins, particularly their native tertiary and quaternary structure are important for such proteins' activity. The term "activity" is defined as a physiological process or participation in a biochemical reaction, e.g. the ability of an enzyme to cause a modification of substrate. In order to be active, proteins should retain their native structure. Proteins will not be active once they are subjected to forces that tend to disrupt their native structure physically or chemically and, thereby, denature them. Forces that can denature proteins include, but are not limited to, pH changes, detergents and excessive heating. (See, e.g., Biological sciences, 4th edition, Keton and Gould, eds., chapter 3, e.g. p.67, 1986).

4. Soybeans were first cultivated in Asia as a crop rotation material (circa 1134-246 BC) (Soybeans, Chemistry, Technology and Utilization, Edited by K. Liu, page 1-3, history, 1999.). During this time, soybeans were not consumed as food due to serious gastric distress that resulted from eating the raw bean (Soybeans, Chemistry, Technology and Utilization, Edited by K. Liu, page 1-3, history, 1999.). Once precipitation and fermentation techniques were developed, Soybeans were incorporated into the Chinese diet. Heat

inactivation of proteins present in the soybean such as Soybean Trypsin Inhibitor ("STI") and Bowman-Birk Inhibitor ("BBI")during soybean processing renders soybeans edible (reviewed in (Wallace et al., 1971), (Kwok and Niranjan, 1995). It was known that the observed gastric distress is the result of inhibition of protein digestion by trypsin and other digestive proteases by the potent serine protease inhibitors, STI and BBI.

5. The negative effects of native STI to the digestive system are heavily documented. Silva et al (1986) documented morphological alterations of small intestinal epithelium, caused by feeding calves with non-denatured soy proteins. Pancreatic enlargement induced by orally ingested STI was documented and studies by Wilson et al (1978) and Krogdahl et al (1979) and reviewed by Flavin (1982). Liener (1983) summarized similar observations in a publication entitled "Naturally occurring toxicants in foods and their significance in the human diet".

6. These studies and others state that STI should be inactivated when soybeans are processed for nutritional use. Numerous studies were conducted to evaluate the effect of processing conditions on the trypsin inhibitory activity and digestibility of various soy preparations (e.g. Wallace et al, 1971, reviewed in Kwok et al, 1995). Today, soy products marketed for nutritional use are processed (e.g. pasteurized, fermented, or cooked) in order to inactivate STI (see e.g. the book: Soybeans, chemistry, technology and utilization, K. Liu, Ed, 1999).

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: February 8th 2002

K. M. Martin

Katharine Martin

MAR 1 8 2002
TRADEMARK OFFICE

DRAFT – 12/11/2001

Docket No. JBP 438

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DECLARATION OF KATHARINE MARTIN

I, Katharine Martin, hereby declare:

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Date: February 8th 2002

K. A. Martin

Katharine Martin